GENETICS OF DNA REPLICATION AND HOMOLOGOUS RECOMBINATION IN ARABIDOPSIS

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Summary

The integrity of genetic information of each cell is constantly subjected to various threats, originating from environmental and endogenous sources. Metabolic by-products and complex DNA-involving molecular processes such as transcription and replication comprise a high intrinsic mutagenic potential. Although these DNA sequence alterations contribute substantially to the evolution of species, they may primarily be detrimental to biological functions and the survival of a cell or, as a cause of mammalian cancer, even to the whole organism. Many evolutionarily conserved molecular machineries control, orchestrate and execute faithful repair of the damaged DNA, ensuring the integrity of the genome prior to its transmission into the next cellular or sexual generation.

Among those machineries, homologous recombination repairs one of the most deleterious DNA lesions - the double-strand breaks - in an accurate fashion, engaging a homologous sequence as template. Alternatively, these breaks are sealed by non-homologous end-joining, which is an error-prone pathway but nevertheless used preferentially in somatic cells of plants and other higher eukaryotes. Thus, the employment of either repair mechanism greatly impacts the genome integrity of cells and is regulated by factors such as cell cycle phase, chromatin structure and availability of the respective repair proteins. Although homologues for most of the repair and replication proteins can be identified in plants, the current knowledge about these molecular pathways and their contribution to genome stability of plant lags far behind other model organisms. In recent years, several repair-related Arabidopsis genes were characterised by reverse genetics, whose outcome suggested a functional conservation of these pathways. This approach could not elucidate the reasons for the prominent exploitation of end-joining to repair double-strand breaks in somatic cells; this may result in substantial alteration of the genetic information in cells, which potentially form the germline of plants.

The development of an artificial reporter system facilitates the *in planta* assessment of the rare homologous recombination events. This allows the genome-wide screening for plant factors that influence the frequency of somatic homologous recombination. The application of this genetic tool resulted in the isolation of an *Arabidopsis thaliana* mutant plant with a moderately increased frequency of intra-molecular homologous recombination. In this mutant line the structure of multiple genes is altered: among them, genes predicted to be a DNA polymerase and a DNA-

dependent ATPase. By genetic means, the dominant mutation responsible for the increased homologous recombination level could be assigned to the DNA polymerase gene: the analysis of allelic mutations and the suppression of the phenotype by the ectopic expression of the polymerase gene confirm the causality between this mutation and the homologous recombination phenotype. The mutated gene encodes for the catalytic subunit of the DNA polymerase δ holoenzyme (POLδ1), which is implicated in multiple aspects of DNA metabolism such as genome replication and most of the DNA repair pathways. The inhibition of cell division in embryos with homozygous *polδ1* mutations underlines the essential function of POLδ1 in replicative DNA synthesis. Moreover, lowered expression of *POLδ1* results in severe developmental aberrations and in genomic instabilities, which are reflected by the frequencies of homologous recombination.

Stalled and collapsed replication forks due to DNA lesions or lack of replication factors trigger cell cycle arrest and apoptosis, avoiding an unbalanced cellular division with deleterious sequence loss. In order to prevent this, molecular mechanisms have evolved, which stabilise the replication fork and promote the resuming of DNA synthesis by a homology-dependent interaction of parental and nascent DNA strands, mediated by proteins of the recombination machinery. Little was known about such mechanisms in Arabidopsis but findings presented in this work provide evidence for an evolutionary conserved function of these processes in plant genome replication. Interfering with S-phase DNA synthesis by chemical inhibition results in an increase of intra-molecular but not of inter-molecular homologous recombination frequency and a similar specificity is observed for *polδ1* mutant alleles. This suggests that hampered or slowed down DNA replication leads to arrested replication forks and the formation of aberrant DNA structures. In order to continue DNA replication, fork reversal and recombination between homologous sequences of the sister-chromatids are engaged, presumably leading to the increased homologous recombination frequencies observed in the Arabidopsis *polδ1* mutant plants.

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Chapter 1 Introduction

1.1 The code of life: a short history about a molecule

In the middle of the 19th century two independent studies were published, which could be considered the starting points of modern molecular biological science. Gregor Mendel's work [1866] with plant "hybrids" proposed that characteristics of both parental plants were transmitted to their offspring, implying a "complete union of the elements in the reproductive cells". The assessed visual phenotypes of parents were passed on to their offspring in a dominant fashion, keeping the appearance of one parent in the following generations or in a recessive fashion, becoming evident only in a subset of plants in the F2 generation. These pioneer experiments proposed the existence of phenotypic information, which was stable and transmissible from generation to generation. Nevertheless, the phenotypes of different parental origin could be combined, suggesting a certain flexibility of this system generally termed as "transformation of a species into another". The work of Charles Darwin [1859] about the formation and evolution of species anticipated that the information for characteristics of a given species cannot only be recombined by sexual reproduction but also influenced by external factors. The challenging environment was proposed to alter the characteristics of a species, which was subjected to "natural selection" by the environment, resulting in a distinct race that can eventually evolve to a novel species. Although the concept of evolution and of genetic heredity were not mutually exclusive, a synthesis of them was only postulated much later by Sewall Wright [1931].

Extensive cytological work on the chromosomes and their behaviour described the duplication and equal redistribution of them in cell division [Strasburger, 1875; Flemming, 1882; van Beneden, 1883]. Finally, these observations and the reduction of chromosomes in meiosis led to the conclusion that they are the carriers of hereditary properties [van Beneden, 1887; Weismann, 1889; Boveri, 1890]. In the following decade, the independent rediscovery of Mendel's studies by several researchers initiated modern genetics [Correns, 1900; de Vries, 1900; von Tschermak, 1900], giving rise to numerous publications and to the creation of the genetic terminology (e.g. gene, genotype, mutation, heterozygosis...) [Bateson, 1909; Johannsen, 1909]. A number of pioneering genetic studies was performed by Thomas Morgan [1915] in the fruit fly *Drosophila melanogaster* developing the first genetic maps. In 1931, Barbara McClintock and Curt Stern established the link between genetic and cytological

crossing-overs in corn *Zea mays* and Drosophila, respectively [Creighton and McClintock, 1931; Stern, 1931].

However, the nature of the above proposed hereditary properties or "genes" remained unsolved for a long time. After the discovery of the nucleic acid by Friedrich Miescher [1871; 1897; reviewed in: Dahm, 2005] and the analysis of its chemical composition [Kossel, 1883-1903], this substance was hypothesised to be the carrier of the genetic information. Walter Sutton and Theodor Boveri independently proposed the "chromosomal theory of inheritance" suggesting a link between the nuclear chromatin, either of its protein or the nucleic acid component, and the hereditary properties [Sutton, 1903; Boveri, 1904]. Pioneer experiments were performed by Fred Griffith, who first showed the "transformation" of the genetic material of bacteria. This work was the basis for the final evidence, provided by Avery *et al.* [1944] and by Hershey and Chase [1952] who showed the horizontal transfer of features between *Pneumococcus* strains by deoxyribonucleic acid-containing (DNA) cell fractions and the solely requirement of DNA as genetic material for a viral infection, respectively.

The description of the structure of the DNA molecule [Watson and Crick, 1953] and the deciphering of the genetic code [Crick, 1962] initiated the era of molecular biology. In recent years, these advances peaked in the sequencing of the whole genome of several organisms from bacteria to human (e.g. *Escherichia coli* [Blattner *et al.*, 1997], *Saccharomyces cerevisiae* [Mewes *et al.*, 1997], *Caenorhabditis elegans* [*C.elegans* Sequencing Consortium, 1998], *D.melanogaster* [Adams *et al.*, 2000], *Arabidopsis thaliana* [Arabidopsis Genome Initiative, 2000] and *Homo sapiens* [International Human Genome Sequencing Consortium, 2004]). The combination of genetic and physical maps greatly facilitated the discovery of genes and the elucidation of their biological functions.

DNA combines the above mentioned features of being replicable in a semi-conservative fashion still allowing certain flexibility to account for evolutionary aspects. The discovery of the double-helical structure of DNA and of the genetic code greatly influenced the deciphering of the cellular mechanism for the amplification and the maintenance of the genetic information, keeping many scientists busy over the last two centuries [reviewed in historical terms in: Alberts, 2003; Friedberg, 2003]. Being the memory of vital function, this fascinating and unique molecule and the molecular processes dealing with it are going to be the protagonists of the work presented here. Many aspects of DNA metabolism such as DNA replication, genome maintenance and flexibility will be discussed here.

1.2 Interactions of plants with their environment

All organisms constantly are in intimate contact with their environment, which provides the essentials for life but may also be detrimental. Suitable to their sessile lifestyle and dependency on solar light, plants have evolved a remarkable morphological, developmental and genomic plasticity [Walbot, 1996; Walbot and Evans, 2003]. Environmental stimuli and impacts such as availability of light, water and nourishment, as well as the presence of various pathogens [Jackson and Taylor, 1996] or cyto- or genotoxic compounds influence the physiology of plants to a great extent.

1.2.1 Genome evolution

The environment has an enormous impact on the evolution of the organisms and their genomes: it selects for advantageous traits but also provokes genome alterations. Over millions of years this continuous mutation and selection cycle has given rise to the present wide variety of phenotypically distinct species and the diversity in sequence and organisation of their genome. Duplications of genes and even of entire genomes, gain and loss of DNA as well as modifications on the sequence level were contributing to this process [discussed in: Arabidopsis Genome Initiative, 2000; Vision *et al.*, 2000]. The triggers for the alterations of the genetic information can be “home-made” by unfaithful DNA metabolism or by endogenous genotoxic compounds but they can also be of external origin (see below and page 54).

The life strategy of plants is well adapted to the environmental threats to their genomes; plants have evolved a series of peculiarities in order to deal with them [reviewed in: Walbot, 1996; Walbot and Evans, 2003]. In contrast to animals, in which the germline is defined early in embryogenesis, reproductive cells of plants are differentiated relatively late in development; the switch to reproduction is triggered by environmental and physiological signals [Simpson and Dean, 2002; Weigel and Jurgens, 2002]. This plant-specific feature increases the possibility of transmitting somatic genome alteration into the next generation [McClintock, 1984]. On the other hand, it also allows “on-plant” selection for mutations, which are potentially advantageous in a changing environment or harmful for the stem cell linage. In the haploid life phase recessive deleterious mutations are removed very efficiently [Gu *et al.*, 2003]. The prolonged haploid gametophytic stage, double-fertilisation as well as parent-of-origin effects are proposed to participate in checking the integrity of the genome, in order to eliminate deleterious somatic mutations [discussed in: Walbot and Evans, 2003].

The frequent occurrence of endoreduplication is another plant-specific feature, which might influence genome flexibility [reviewed in: Larkins *et al.*, 2001; Sugimoto-Shirasu and Roberts, 2003; Kondorosi and Kondorosi, 2004]. Endoreduplication defines a mechanism of genome replication without cytokinesis and redistribution of chromosomes, resulting in nuclei of higher DNA content. This phenomenon increases the metabolic potential of cells but may also buffer or mask the detrimental effects of mutations by providing multiple copies of each gene. Remarkably, the enlarged genome requires more sophisticated mechanisms for replication and maintenance of the DNA; plants are the only eukaryotes that have retained functional homologues of the archaebacterial topoisomerase VI complex [Hartung and Puchta, 2001]. Mutations in both Arabidopsis topoisomerase IV subunits – *SPO11-3* and *TOP6B* –, resulted in dwarf plants with impaired DNA replication and endoreduplication [Hartung *et al.*, 2002; Sugimoto-Shirasu *et al.*, 2002].

1.2.2 Impacts on plant genome stability

Plants have been proposed to offer an excellent bio-monitoring system for environmental factors that influence genome stability when provided with an easily assessable transgenic marker for DNA changes [Kovalchuk *et al.*, 2001a]. So far, a series of Arabidopsis and tobacco lines were generated, which are able to “measure” genome stability or instability, respectively. The restoration of a functional marker gene at a given locus reflects either HR events [Schuermann *et al.*, 2005], point mutations [Kovalchuk *et al.*, 2000a] or frame shift mutations [Alou *et al.*, 2004]. As a marker for genome instability upon exposure to exogenous stimuli, the level of HR events in somatic cells was most frequently analysed. As expected, the plants reacted with increased genome instability to known genotoxic and mutagenic factors such as ionising radiation (IR), UV light, the crosslinking agent mitomycin-C (MMC) or the reactive oxygen species (ROS) [Tovar and Lichtenstein, 1992; Lebel *et al.*, 1993; Puchta *et al.*, 1995b; Kovalchuk *et al.*, 1998; Kovalchuk *et al.*, 1999; Kovalchuk *et al.*, 2000b; Ries *et al.*, 2000a; Ries *et al.*, 2000b; Filkowski *et al.*, 2004b; Molinier *et al.*, 2004c]. Application of other abiotic environmental stresses also yielded elevated levels of recombination events: temperature and high salinity [Lebel *et al.*, 1993] or heavy metals [Kovalchuk *et al.*, 2001b]. The exact molecular mechanisms behind this stress-induced genomic instability are not known but it can be speculated that cells respond to them by physiological changes, which directly or indirectly harm the DNA (Figure 1).

Similarly to exposure to directly damage-inducing factors, this leads to more DNA repair activities and reduced genome stability.

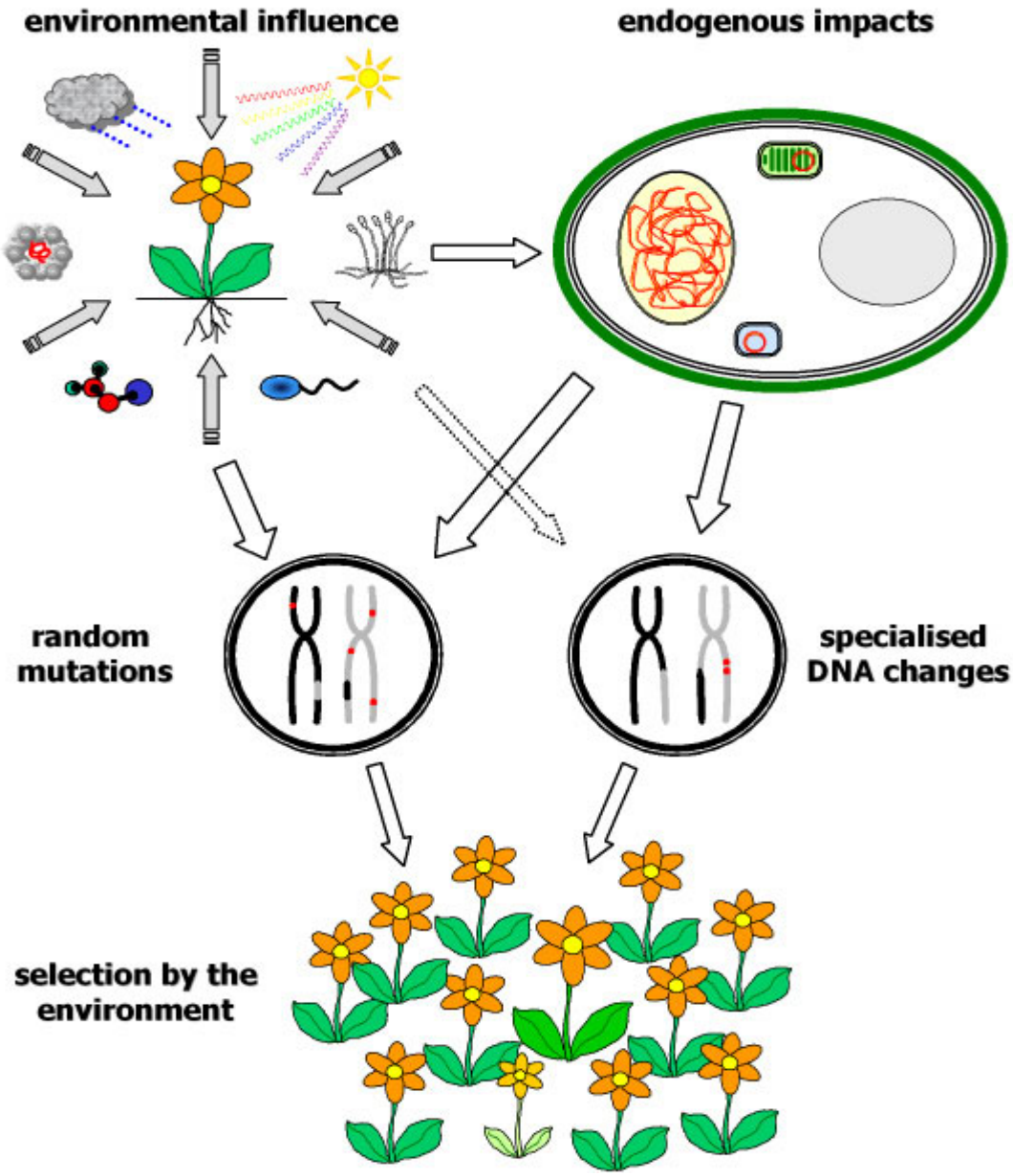


Figure 1: Impacts on plant genomes
The environment triggers physiological changes in plants and greatly influences their development and morphology. Environmental factors can also provoke DNA lesions, either directly or indirectly, by the chemical or physical interaction with DNA or by the stimulation of cells to form genotoxic compounds. Normal cellular metabolism is responsible for the majority of the random DNA lesions. Some DNA changes are induced by specialised cellular mechanisms, provoking advantageous sequence alterations and recombinations. Finally, all genome alterations are subjected to selection by the environment.

Higher eukaryotic organisms possess defence mechanisms against pathogenic and quickly evolving micro-organisms. The mammalian immune system adapts to new

races of pathogens by the formation of novel antibodies by controlled modification of genetic information (see below). It is tempting to speculate about similar mechanisms that might exist in plants and indeed, rapid generation of new resistance genes with novel specificities was reported for the corn Rp1 complex [Richter *et al.*, 1995; Ramakrishna *et al.*, 2002], the rice Xa21 family [Song *et al.*, 1997] and the tomato Cf-4/9 locus [Parniske *et al.*, 1997; Van der Hoorn *et al.*, 2001; Wulff *et al.*, 2004]. Genome analysis of Arabidopsis revealed the clustered organisation of disease resistance genes belonging to the class of NBS-LRRs [reviewed in: Richly *et al.*, 2002; Meyers *et al.*, 2003]. They originate from tandem and segmental gene duplication. Recombination events between these homeologous sequences often result in the generation of new resistance genes [Baumgarten *et al.*, 2003]. In a resistance gene-independent locus, enhanced somatic genome instability was reported in Arabidopsis and tobacco plants with a transgene-based HR reporter upon inoculation with pathogenic oomycete *Peronospora parasitica* [Lucht *et al.*, 2002] or with tobacco mosaic virus, respectively [Kovalchuk *et al.*, 2003]. The same effect on genome stability was obtained by constitutive signalling or by mimicking pathogen attack with a bacterial elicitor [Lucht *et al.*, 2002; Molinier *et al.*, 2004c]. These observations suggest rather an indirect and random impact of pathogen contact on the genome than controlled events. Those may arise from the physiological response to environmental challenges such as transcriptional changes or the production of ROS, which mediate the typical defence reaction of plants called hyper-sensitive response [rewieved in: Heath, 2000; Lam *et al.*, 2001]. Further support is provided by the observation that the genetic instability is also transmitted to uninfected tissue and can be suppressed by radical scavengers [Kovalchuk *et al.*, 2003; Filkowski *et al.*, 2004b]. However, pathogen-induced stress (probably other stresses as well) does reduce genome stability, which facilitates and enhances the creation of advantageous traits and therefore the change to survive in a challenging environment. Bacteria possess a similar mechanism termed “adaptive mutation”, resulting in elevated mutation rates in stressed cells [reviewed in: Foster, 2000; Rosenberg, 2001]. Whether this process happens at random or is directed to genes most likely to provide relief from the stress is still debated. The evolution of disease resistance genes in Arabidopsis could be stimulated in a similar fashion, driven by mutational forces such as unequal recombination, gene conversion, point mutations and transposon activation [discussed in: Leister, 2004].

1.3 Sources of DNA damage and genome alteration

Lesions on the DNA molecule are constantly occurring in every cell. External and internal factors provoke alteration of the genetic information, favoured by the intrinsic reactivity of the chemical DNA constitution (Figure 1). For a human cell, a spontaneous daily loss of 10^4 bases by hydrolysis of the glycosyl bond was estimated [Lindahl and Nyberg, 1972]. Similarly, many miscoding basepairs can occur from natural deamination of cytosine, 5-methyl-cytosine, guanine or adenine [Duncan and Miller, 1980; Lindahl, 1993]. Apart from these spontaneously arising DNA damages, a broad variety of high-energetic radiations and reactive molecules were identified, which may harm the structure or the composition of the DNA. They originate either from the environment or from cellular metabolisms such as oxidative respiration, lipid peroxidation and photosynthesis of plants or from DNA metabolism.

1.3.1 Endogenous causes

Endogenously produced DNA lesions are thought to contribute significantly to the formation of human cancer and might therefore account for the majority of DNA damages [discussed in: Jackson and Loeb, 2001]. The reactive oxygen species (ROS) are metabolic by-products with a high potential to induce dozens of different kinds of oxidative damage on the base or the sugar moiety of DNA [reviewed in: Cadet *et al.*, 1997; Gros *et al.*, 2002]. Cells fight against the inevitable production of ROS by compartmentation of metabolic pathways into organelles such as mitochondria or chloroplasts, by producing ROS scavengers and by enzymatic detoxification of ROS [Finkel and Holbrook, 2000]. However, remaining ROS still result in many types of oxidative DNA lesions such as 8-oxo-guanine (8oxoG), thymine glycol, single-strand breaks (SSB) and many more [Martinez *et al.*, 2003]. The significance of the oxidative metabolism for genome instability was substantiated by a recent work in mice, in which its participation in the creation of endogenous DSBs was shown [Karanjawala *et al.*, 2002]. Arabidopsis plants depleted for some ROS-defensive proteins also exhibited increased genome instability [Ries, 1999; Filkowski *et al.*, 2004a]. Apart from the ROS there are also other small reactive endogenous molecules which could harm DNA. *S*-adenosylmethionine (SAM) is a widely used cofactor for cellular methylation reactions. For a human cell, the estimated number of non-enzymatic methyl-group transfers from SAM to DNA (mostly adenine bases) was about 600 a day [Lindahl, 1993]. Malone-di-aldehyde, a product of lipid peroxidation, was proposed to cause bulky DNA base adducts [Niedernhofer *et al.*, 2003]. These few examples illustrate the genotoxic

impact of endogenous molecules; it is likely that many more remain to be found and described [Lindahl and Wood, 1999].

Cellular processes that involve DNA are potentially mutagenic as well; for instance, increased mutation levels were reported for highly transcribed yeast genes [Datta and Jinks-Robertson, 1995]. Chromatin remodelling and DNA unwinding during replication and transcription may cause structural torsions, which facilitate or even provoke strand breaks. DNA synthesis itself can lead to sequence alterations. Despite proofreading activities of the processive DNA polymerases, replication produces many mispaired bases or extrahelical nucleotides [Hübscher *et al.*, 2002]. In addition, translesion DNA polymerases, which are able to bypass damaged template bases and thus ensure frictionless DNA replication, are of a low accuracy and increase the possibility of sequence alterations [Rattray and Strathern, 2003]. Unfaithful or aborted repair of DNA damage can lead directly to genomic changes or give rise to mutagenic intermediates. Mobile DNA elements are also contributing to genome alteration and evolution. The excision of transposable elements creates DNA strand breaks or their reintegration potentially alters the genome sequences at the recipient locus [reviewed in: Bennetzen, 2000; Gray, 2000]

1.3.2 Exogenous factors

The ROS discussed above do not only arise from cellular metabolism but are also present in the environment. Similarly, many chemical compounds existing in the environment or produced by men have DNA damaging features when taken up by the organisms. They are able to induce a multiplicity of lesion types, ranging from base modifications, bulky adducts to DNA double-strand breaks (DSB) [extensively discussed in several chapters of: Hock and Elstner, 1995].

Among the external DNA damaging factors, ionising radiation (IR) and ultraviolet light (UV) are probably the most important ones. UV-B (280-320 nm) and UV-C (200-280 nm) are components of solar light. The nutritional dependence of most plants on sunlight and their sessile life-form inflict continuous exposure to UV irradiation, which prevalently induces cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) [reviewed in: Friedberg *et al.*, 1995]. Both of these lesions result in distorted DNA structure and are very abundant types of damages; it has been estimated, that strong sunlight provokes about 40,000 damaged sites per hour in a human epidermal cell [Ura and Hayes, 2002].

Organisms are constantly exposed to low doses of ionising radiation, originating from the decay of air-borne and ground radioactivity. Ionising radiation such as X- or γ-rays induces complex clustered DNA damages consisting of oxidative lesion and strand breaks [discussed in: Sutherland *et al.*, 2000]. Due to their complexity these damages are very difficult to repair in an accurate manner.

1.3.3 Induced genome alteration

The causes of genome alteration described above are likely to occur randomly and to have a serious impact on cell survival. However, there are several examples of directed and controlled DNA changes, mostly restricted to specialised cell types. Best know is the process of meiotic recombination, which rearranges the parental genetic information in a random fashion and thereby creates new combinations of traits. Except for the initial induction of DSB, this process virtually requires the same set of enzymes as for the accurate repair of breaks by homologous recombination (HR) in somatic cells (see page 11 and 48).

Mammalian immune response depends on the creation of a wide spectrum of different antibodies. This diversity is enabled by two mechanisms called V(D)J recombination and somatic hypermutation. In V(D)J recombination, pre-existing genes are cleaved by sequence-specific endonucleases and the DNA fragments are subsequently rejoined in a random manner. This process uses the same core enzymes for DSB repair by non-homologous end-joining (NHEJ) and additionally some specialised ones (see page 19) [reviewed in: Gellert, 2002; Roth, 2003]. Upon induction by the presence of an antigene, “fine tuning” of the antibody specificity is mediated by somatic hypermutation, which actively mutates the antibody-coding sequences by deamination of cytosine to uracil and the utilisation of error-prone DNA polymerases [reviewed in: Harris *et al.*, 1999; Rattray and Strathern, 2003]. Other well known examples for targeted genome modifications are the repeat-induced point mutation (RIP) of *Neurospora crassa*, which integrates epigenetic modifications and sequence changes [reviewed in: Galagan and Selker, 2004] or the mating type switch of yeast [Haber, 1998b].

1.4 Repair of damaged DNA

DNA lesions are caused continuously by external as well as internal factors (see above). Their detection and repair is crucial to the function and survival of each cell and as a prerequisite for cancer even for a whole organism. All organisms have therefore evolved a sophisticated molecular network, which integrates DNA damage recognition, signalling and repair mechanisms [reviewed in: Kolodner *et al.*, 2002; Rouse and Jackson, 2002]. A variety of damage sensors are able to detect anomalous DNA structures and to trigger cellular responses such as recruitment of repair proteins, checkpoint activation and cell cycle arrest, transcriptional changes or apoptosis. Depending on cell cycle and nature of the damage, different repair and signalling pathways are involved in its immediate removal [extensively reviewed in: Sancar *et al.*, 2004]. The nature of the DNA lesion generally defines the recognition and the repair by a distinct mechanism (see Figure 2). However, there are also some redundancies and interconnections known which are discussed later.

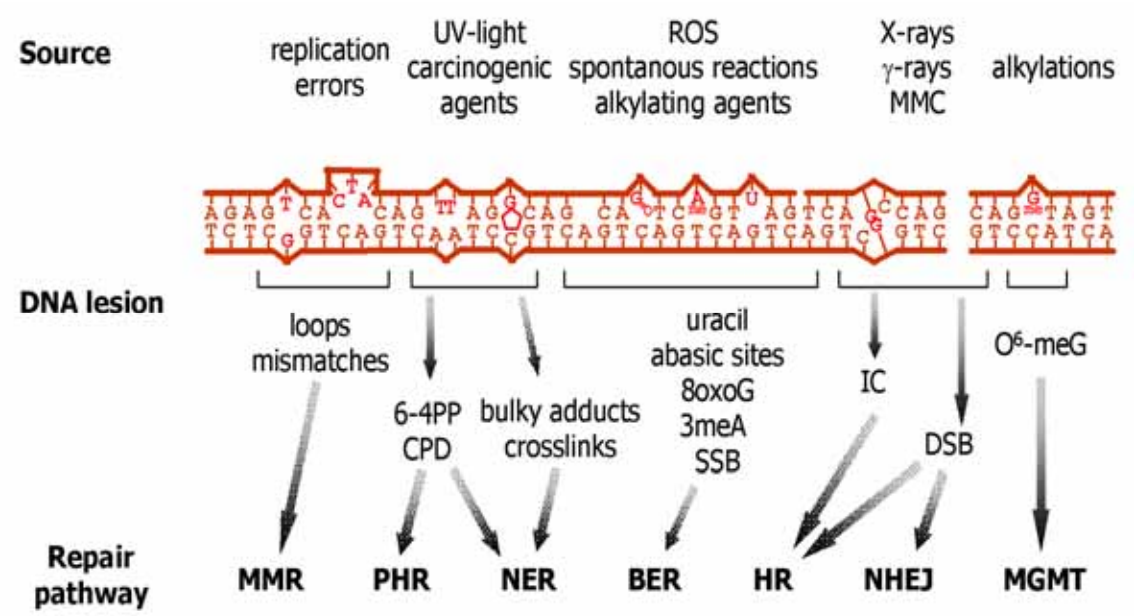


Figure 2: Major lesions of DNA; their causes and their repair
Exo- and endogenous molecules and radiations provoke a wide variety of molecular and structural modifications of DNA. In order to preserve the genetic information, these lesions are recognised and repaired by distinct repair mechanisms: mismatch repair (MMR), photo-reactivation (PHR), nucleotide excision repair (NER), base excision repair (BER), homologous recombination (HR), non-homologous end-joining (NHEJ), O^6-methylguanine-DNA methyltransferase (MGMT). 6-4PP, pyrimidine (6-4) pyrimidone photoproducts; CPD, cyclobutane pyrimidine dimers; ROS, reactive oxygen species; 8oxoG, 8-oxo-guanine; 3meA, 3-methyl-adenine; SSB, DNA single-strand break; IC, interstrand crosslink; MMC, mitomycin-C; DSB, DNA double-strand break; O^6-meG, O^6-methyl-guanine.

1.4.1 Homologous recombination

The term homologous recombination (HR) is generally used for an interaction between DNA molecules of complementary sequence. This mechanism is used for DNA repair andr for meiotic recombination. HR repairs DNA double-strand breaks (DSBs) in a very accurate fashion, using the undamaged sister chromatid or another homologous sequence as template. In yeast and probably in other organisms as well, HR may involve the search for homologous sequences throughout the entire genome [Inbar *et al.*, 2000]. Preferentially, the sequence information for repair of broken DNA is derived from the sister chromatid [Gonzalez-Barrera *et al.*, 2003] but the homologous chromosome (allelic recombination) [Palmer *et al.*, 2003] and homologous sequences anywhere in the genome (ectopic recombination) are used as well [Inbar and Kupiec, 1999; Aylon and Kupiec, 2003]. Genetic, biochemical and structural studies defined the different steps of homologous recombination according the double-strand break repair model (see below): 1) nucleolytic end processing; 2) nucleoprotein-filament formation; 3) homology search and strand invasion; 4) DNA synthesis; and 5) Holliday junction (HJ) resolution [reviewed in: West, 2003; Aylon and Kupiec, 2004].

1.4.1.1 The recombinosome

At sites of DSBs, nucleolytic resection leads to the formation of 3'-protruding single-stranded DNA (ssDNA), which is recognised and subsequently handled by the HR repair machinery (Figure 3). Several yeast nucleases such as Mre11, Exo1 and Rad27 were proposed to participate in the processing of DNA ends [Haber, 1998a; Moreau *et al.*, 2001]. Nevertheless, it is still unclear, which one of these enzymes carry out this step, since even a multiple genetic combination of their mutants did not result in the abolishment of resection [discussed in: Aylon and Kupiec, 2004]. In yeast the ssDNA is immediately coated by the replication factor A (RFA) [Wang and Haber, 2004], which stimulates the binding of Rad52 to the processed DSB [Sung, 1997a; Sugiyama *et al.*, 1998]. The same mechanism was also found for the human homologues [Van Dyck *et al.*, 1999]. The subsequent formation of the typical repair foci depends on the presence of Rad52 [Gasior *et al.*, 1998; Miyazaki *et al.*, 2004]. Recent *in vivo* studies in yeast and human cells suggest a step-wise assembly of recombination factors of the Rad52 epistasis group onto the breaks [Essers *et al.*, 2002; Sugawara *et al.*, 2003; Wolner *et al.*, 2003]. Initially, a nucleoprotein filament with the recombinase Rad51 coating the ssDNA is formed by Rad52-mediated replacement of RFA [Gasior *et al.*, 1998]. In humans there is an additional player in the early steps of HR: the

heterodimeric complex of the breast cancer suppressors BRCA1 and BRCA2 [Moynahan *et al.*, 1999; Moynahan *et al.*, 2001; Xia *et al.*, 2001] that are essential for genome integrity [Xu *et al.*, 1999; Yu *et al.*, 2000]. BRCA2 interacts directly with RAD51, is required for the formation of repair foci at the sites of DNA damage and stimulates the homologue pairing and strand exchange activity of RAD51 *in vitro* [Yuan *et al.*, 1999; Yang *et al.*, 2002]. The BRCA complex is suggested to have a scaffolding function: it keeps RAD51 inactive until DNA damage occurs. Upon insult it promotes release and loading to ssDNA of RAD51[discussed in: West, 2003].

The complex with Rad52 and the Rad51-coated filament is then searching for sequence homology on the template DNA strand and subsequently invades the double-stranded DNA (dsDNA) to form a joint molecule [Miyazaki *et al.*, 2004]. This crucial step of HR is facilitated by a series of other proteins. The heterodimer of yeast Rad55/Rad57 stabilises the nucleoprotein filament and stimulates the strand exchange activity of Rad51 [Sung, 1997b], promoting the strand invasion and heteroduplex formation [Aylon *et al.*, 2003]. Similar functions were assigned to the human Rad51 paralogs: XRCC2, XRCC3, Rad51B-D [van Gent *et al.*, 2001].

The strand invasion of the nucleoprotein filament also requires Rad54, a member of the SNF2 dsDNA-dependent ATPase family [Tan *et al.*, 2003]. It interacts directly with Rad51 [Petukhova *et al.*, 1998], facilitates strand invasion and pairing as well as heteroduplex expansion by its chromatin remodelling activity [Alexiadis and Kadonaga, 2002; Alexeev *et al.*, 2003; Jaskelioff *et al.*, 2003]. Interestingly enough, the yeast genome encodes a Rad54-like protein Rdh54 (Tid1), mutants of which specifically interfere with allelic HR in mitotic as well as meiotic cells [Shinohara *et al.*, 1997]. Rhd54 may therefore promote strand invasion between homologous chromosomes, most importantly in meiotic HR (see page 48) [Shinohara *et al.*, 2000].

Displacement of Rad51 from the nucleoprotein filament was also reported for the 3'-5' DNA helicase Srs2 [Krejci *et al.*, 2003; Veaute *et al.*, 2003]. Genetic evidence suggested that Srs2 is functionally redundant to Sgs1 [Gangloff *et al.*, 2000], a member of the RecQ DNA helicase family, to which also the human Bloom (BLM) and Werner (WRN) syndrome proteins belong. Humans carrying mutations in these genes exhibit chromosome aberrations and cancer-predisposition [reviewed in: van Brabant *et al.*, 2000; Hoeijmakers, 2001]. The slow growth phenotype of yeast *sgs1*/*srs2* double mutants can be suppressed by the depletion of any recombination gene, suggesting a function of Sgs1 and Srs2 as negative regulators of HR [Gangloff *et al.*, 2000; Ira *et al.*, 2003]. These DNA helicases may control the nucleoprotein invasion

and homology search, the switch to DNA synthesis or the formation of aberrant HR intermediates [Aylon and Kupiec, 2004].

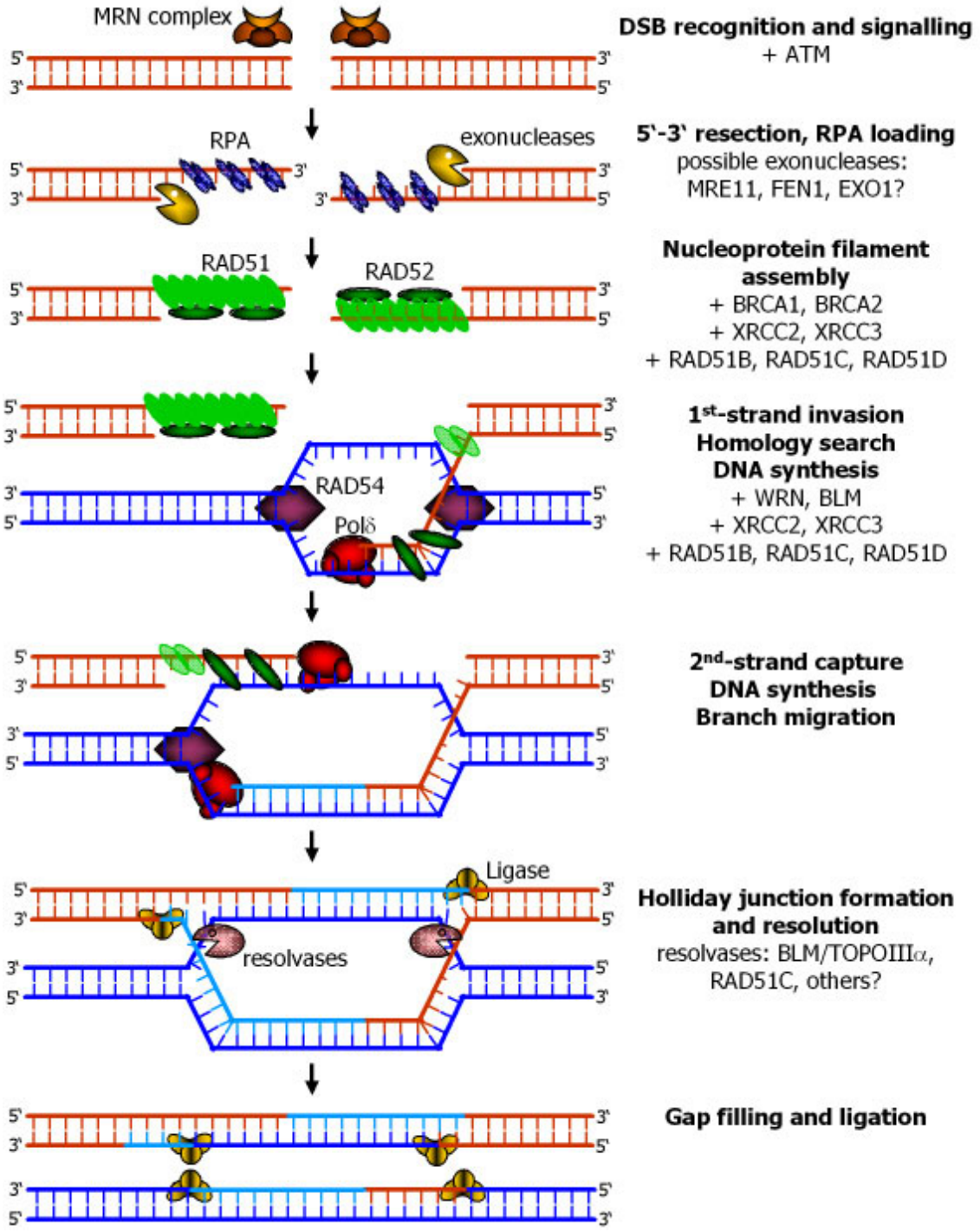


Figure 3: The mechanism of homologous recombination
Schematic representation of the molecular interaction between DNA strands and the enzymatic machinery in the homologous recombination DSB repair pathway, derived from genetics and biochemistry of budding yeast and mammals (left column). Key steps of HR are mentioned in the right column, as well as additional proteins, which are proposed to participate. The damaged and the undamaged dsDNA are depicted as brown and blue lines, respectively. Light blue lines indicate newly synthesised DNA, using the undamaged chromatid as template.

Rad54 was also shown to be able to remove Rad51 from the ssDNA *in vitro*, allowing base-pairing and formation of the heteroduplex [Solinger *et al.*, 2002].Once the invading strand is paired with the template, DNA synthesis is initiated and branch migration starts. Results of experiments employing a series of temperature sensitive mutants of the main replication DNA polymerases and their accessory factors (see page 43) allowed the proposal that the whole replication complex is required for HR, implying the need for leading and lagging strand synthesis [Holmes and Haber, 1999].

However, a recent publication questioned this observation, demonstrating the requirement of only leading strand synthesis by the DNA polymerases δ and ε [Wang *et al.*, 2004]. In this later step of HR, the ATPase Rad54 was suggested to play a role in branch migration during DNA synthesis [Tan *et al.*, 2003]. Rad52, which in contrast to Rad51 remains associated with the interacting chromatids, may promote the re-annealing of the newly synthesised DNA with the second broken arm.

Finally, the HJs have to be resolved and the DNA gaps ligated. At least, 3 independent resolvase activities were described in the literature: the Mus81/Eme1 nuclease of fission yeast [Boddy *et al.*, 2001; reviewed in: Hollingsworth and Brill, 2004]; the biochemically defined human resolvase A, which probably corresponds to RAD51C [Constantinou *et al.*, 2001; Liu *et al.*, 2004]; and a budding yeast complex of Sgs1 and the topoisomerase 3 (Top3) [Fabre *et al.*, 2002] as well as their human homologues BLM/TOPOIIIα [Wu and Hickson, 2003].

1.4.1.2 Homologous recombination: types and models

A prototypic model for the interactions of the two broken ends and the template strand, DNA synthesis and resolution was proposed by Szostak [1983]: the double-strand break repair (DSBR) model. Since then, a variety of models for HR were suggested (Figure 4), supported by molecular evidence [extensively reviewed in: Paques and Haber, 1999; Krogh and Symington, 2004].

Double-strand break repair (DSBR) model: This model is based on the concept of Holliday [1964] for meiotic gene conversions. One of the extensive stretches of 3'-protruding ssDNA at the DSB invades the homologous duplex and serves as a primer for DNA synthesis (Figure 4A). A D-loop is created by strand invasion and branch migration, which pairs with the second strand and also primes DNA synthesis. Upon gap filling and ligation, recombination intermediates with double Holliday junctions (dHJ) are formed. Alternative resolution of the HJs yields crossover and non-crossover products but necessarily invokes the exchange of some DNA stretches between the

homologous sequences (see Figure 4). This model explains the features of meiotic recombination quite well but recent studies propose the existence of other HR intermediates [Allers and Lichten, 2001], explaining the higher frequency of non-crossover events (see below).

Synthesis-dependent strand-annealing (SDSA) model: This model accounts for a lower frequency of crossovers for meiotic as well as mitotic HR than predicted. One of the ssDNA ends invades the template DNA and becomes elongated by DNA synthesis (see Figure 4B). Subsequently, the strand is released from the heteroduplex, pairs with the second resected strand and the remaining gaps are filled and sealed. Alternatively, both strands are elongated, one invades the heteroduplex and the other captures the free strand of the D-loop. Notably these models do not invoke the formation of HJ. Gene conversion events are best described by them since no crossovers and no sequence exchanges happen.

Single-strand annealing (SSA): This mechanism reflects a way of repairing DSBs resembling NHEJ (Chapter 1.4.2) but involves homologous sequences, positioned as direct repeats on the same chromosome. This process leads to the deletion of one of the repeats and the intervenient sequences and is therefore mutagenic. Both ends of the DSB are resected and the homologous sequences are paired (see Figure 4C). Unpaired ssDNA is removed by nucleases and the gaps are filled and finally sealed. In yeast, the repair of DSBs by SSA requires only the presence of Rad52 for homologous pairing but no other HR protein [Ivanov *et al.*, 1996]. Furthermore, the requirement of Rad52 for SSA decreases with the length of homology of the repeats. On the other hand, the removal of the 3' overhanging flap structure often formed in SSA depends on the structure-specific Rad1 endonuclease [Paques and Haber, 1999].

Break induced replication (BIR): Whereas the above HR models involve short patch events, BIR explains long conversion tracts in mitotic cells [Kraus *et al.*, 2001]. One-ended ssDNA invades the duplex, basically creating a replication fork (see Figure 4D). The assembly of a functional replication complex results in the duplication of virtually whole chromosome arms. The BIR mechanism is proposed to carry out a biologically relevant role in telomere maintenance [Lundblad and Blackburn, 1993] and depends on the presence of HR proteins [reviewed in: Le *et al.*, 1999; Davis and Symington, 2004].

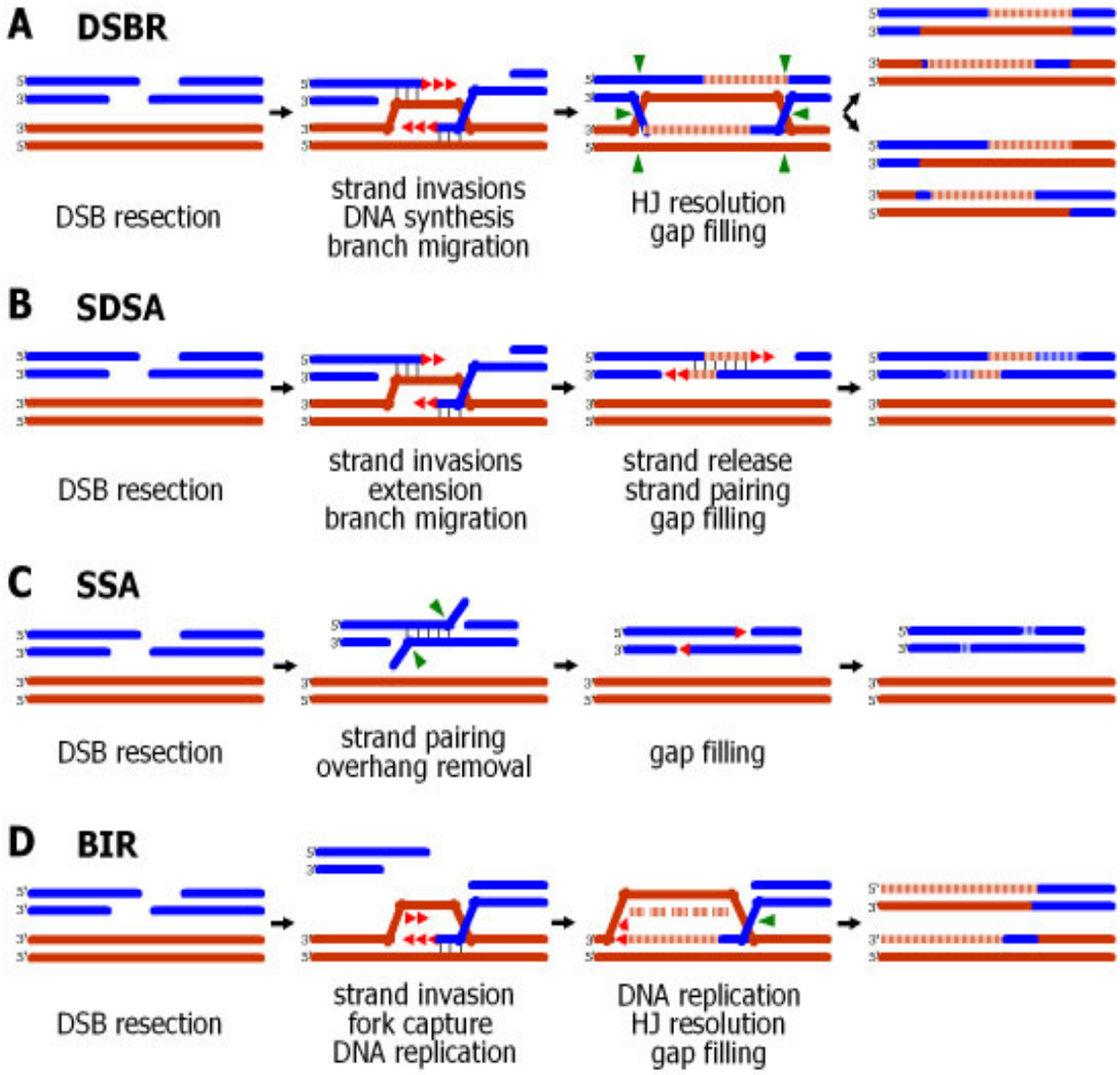


Figure 4: The main models of homologous recombination
Schematic depiction of some of the proposed homology-based molecular interaction of DNA strands to repair DSBs. **A.** The classical double-strand break repair (DSBR) model by Szostak [1983]. **B.** An example of the synthesis-dependent single-strand annealing (SDSA) model that involves the invasion of both DSB ends. **C.** The mutagenic single-strand annealing (SSA) model. **D.** Break-induced replication (BIR) that leads to the assembly of a replication fork at the site of strand invasion. For a more detailed description of the process, see above. Black lines indicate the pairing of homologous sequences. Arrowheads mark the direction of DNA synthesis (red) or sites of endonucleolytic processing (green). Patterned lines in the respective colour of the template strand represent newly synthesised DNA.

In yeast, the vast majority of meiotic and mitotic recombination events can be explained by the models introduced above. Frequency and segregation of heterozygous markers indicating crossovers and gene conversion events in meiosis correlate well with HR following the DSBR and SDSA models. In addition, HJ structures as consequence of DSBR were proven to correspond indeed to the cytologically observed crossover events [Schwacha and Kleckner, 1995], about 100 of which were seen in

yeast meiotic chromosomes [Paques and Haber, 1999]. The fact that crossovers are also observed in plant meiosis is an implication for the existence of meiotic HR events according to the two main models, although the number of crossovers is considerably lower than in yeast (e.g. about 10 in Arabidopsis) [Creighton and McClintock, 1931; Copenhaver *et al.*, 1998]. The relatively low meiotic recombination frequency of Arabidopsis could be due to sequence composition, to chromatin structure [Petes, 2001] or to the size of chromosomes. This does not necessarily imply an inefficient HR machinery, since a reciprocal correlation between number of crossovers, and the lengths of the yeast chromosomes was shown [Kaback *et al.*, 1992].

The analysis of Arabidopsis crossover interference revealed more than 10 times more induced meiotic DSBs than crossovers and supported the notion of an additional interference-independent crossover pathway [Copenhaver *et al.*, 2002]. However, the analysis of recombination events on the basis of functional restoration of transgenic markers yielded mostly ectopic recombination by non-reciprocal crossovers [Jelesko *et al.*, 1999; Jelesko *et al.*, 2004] and allelic recombination by gene conversion [Molinier *et al.*, 2004b]. These observations emphasise a similar fashion of meiotic recombination in plants as in other organisms, presumably following the DSBR and SDSA models of HR.

In contrast to meiotic cells of plants, the use of HR to repair somatic DSBs preferentially happens in a manner, which can be explained by the SDSA model involving the invasion of one or both DNA ends into the homologous duplex, or by the SSA model, when homologous sequences are present nearby [reviewed in: Gorbunova and Levy, 1999; Puchta, 2005]. Several studies in which DSBs were induced by transposon excision or by endonucleases contributed to the current understanding of intra-molecular recombination in plants [Athma and Peterson, 1991; Chiurazzi *et al.*, 1996; Xiao and Peterson, 2000; Siebert and Puchta, 2002]. An about 5 times more frequent repair of induced DSB by the mutagenic SSA pathway was estimated in direct comparison with the conservative SDSA model [Orel *et al.*, 2003]. Unlike other organisms plants depleted for the NER endonuclease RAD1 (see page 28) exhibited an enhanced sensitivity to ionising radiation and were shown to be impaired in HR by SSA, supporting the importance of this pathway in DSB repair [Dubest *et al.*, 2002].

1.4.1.3 Homologous recombination genes of plants

The fully sequenced and annotated genome of Arabidopsis revealed the presence of putative orthologues or homologues of almost all genes that play a role in yeast or human HR [Arabidopsis Genome Initiative, 2000; Hays, 2002]. Some of them were recently cloned and characterized (Table 1), mostly for their activity on genotoxic challenges or their involvement in meiotic HR (see page 48). Surprisingly, a homologue of Rad52, which is the only yeast protein required for virtually all homology-based repair mechanisms [Paques and Haber, 1999], could not be found. However, plants do perform meiotic as well as somatic HR (see above), which suggests the presence of a functional equivalent of Rad52 that remains to be found. So far, there is only one report about altered somatic recombination frequency of a mutant in described HR genes; *rad50* plants were shown to be hyper-recombinogenic [Gherbi *et al.*, 2001] and hypersensitive to methyl methanesulfonate (MMS) [Gallego *et al.*, 2001], supporting the role of RAD50 in DNA repair. Similarly, an increased sensitivity to MMS as well as to X-ray was shown for *mre11* plants [Bundock and Hooykaas, 2002]. Since Rad50 and Mre11 are involved in both the HR and the NHEJ repair pathways, these findings are in favour of the concept of their competition in DSB repair, NHEJ being more frequently used and therefore more affected in these mutants.

Arabidopsis	Human	Budding yeast	Function
MRE11	MRE11	Mre11	End processing
RAD50	RAD50	Rad50	End processing
-?	NBS1	Xrs2	End processing, signalling
?	RPA	RFA	ssDNA coating
RAD51	RAD51	Rad51	ssDNA coating, strand invasion
-	RAD52	Rad52	Nucleoprotein filament formation, strand invasion
?	XRCC2	Rad55	Nucleoprotein filament formation
XRCC3	XRCC3	Rad57	Nucleoprotein filament formation
?	RAD51B	-	Nucleoprotein filament formation
RAD51C	RAD51C	-	Nucleoprotein filament formation, HJ resolution
?	RAD51D	-	Nucleoprotein filament formation
BRCA1	BRCA1	-	Signalling?
BRCA2	BRCA2	-	ssDNA coating, strand invasion?
?	RAD54	Rad54	Strand invasion between sister chromatids
?	?	Rhb54	Strand invasion between interhomologues
?	POLδ/ε	POLδ/ε	DNA synthesis
-	-	Srs2	Strand invasion, nucleoprotein filament formation
RecQ-like/?	BLM/TOPOIIIα	Sgs1/Top3	HJ resolution, RAD51 removal
?	?	Msm4/Mus81	HJ resolution

Table 1: The core proteins of repair by homologous recombination
Described homologous proteins of Arabidopsis, humans and budding yeast and their proposed functions in HR. ?, putative candidate is found in the genome but is not yet described. –, absent homologue.

Rad51-related proteins of yeast and humans are essential for the initiation and progression of HR (see above). The Arabidopsis homologue of Rad51 and its paralogues were described as well [Osakabe *et al.*, 2002]. Plants mutated in *RAD51* [Li *et al.*, 2004b] as well as in *XRCC3* [Bleuyard and White, 2004] exhibited severe defects in meiotic recombination (see page 48), whereby *xrcc3* plants revealed a more pronounced sensitivity to DNA cross-linking and DSB-inducing agents, suggesting a function in somatic repair in addition.

The transcriptional level of the Arabidopsis *BRCA1* gene is induced by γ-ray and in floral tissue [Lafarge and Montane, 2003]. RNAi-repression of both BRCA2 homologues yielded a meiotic phenotype [Siaud *et al.*, 2004] (see page 48). These findings underline their possible roles in the regulation of somatic and meiotic HR of plants. Seven putative homologues of RecQ DNA helicases can also be found in Arabidopsis [Hartung *et al.*, 2000]. Members of this protein family were reported to have several functions in HR. RecQsim, named according to its plant-specific domain organisation, partially suppressed MMS hypersensitivity of yeast *sgs1* mutants [Bagherieh-Najjar *et al.*, 2003]. Although no direct effect on HR was shown, this functional conservation of the HR suppressors of the RecQ family is remarkable.

1.4.2 Non-homologous end-joining

Non-homologous end-joining (NHEJ), sometimes also termed illegitimate recombination, is a quick and efficient repair mechanism for deleterious DNA double-strand breaks, conserved in all organisms (Table 2) [reviewed in: Critchlow and Jackson, 1998; Lieber *et al.*, 2003; Aylon and Kupiec, 2004]. The Ku70/80 complex is normally located at the ends of chromosomes [Gravel *et al.*, 1998], where it maintains the length and integrity of telomeres. Upon DSB induction Ku70/80 becomes relocated to the site of damage [Martin *et al.*, 1999] and binds to the DNA ends, independent of their sequence and terminal structure (Figure 5) [Boulton and Jackson, 1998]. It was suggested that the Ku heterodimer has a role in bridging and juxtaposing DNA ends [Pang *et al.*, 1997; Feldmann *et al.*, 2000] as well as in the recruitment of other proteins [Nick McElhinny *et al.*, 2000; Chen *et al.*, 2001]. However, a bridging function was also proposed for the human DNA-dependent protein kinase (DNA-PK) [Yaneva *et al.*, 1997], which can be found neither in yeast nor in plants. For DNA-PK it was reported that the strength of interaction with Ku70/80 as well as its activation depends on the terminal structure of DNA, stimulated by the presence of longer ssDNA stretches [Hammarsten *et al.*, 2000]. DNA-PK may therefore have additional functions

in DSB signalling and the recruitment of end processing proteins. The lack of DNA-PK and other factors could explain why NHEJ in plants is more prone to errors than it is in other organisms, often accompanied with large insertions and deletions [Gorbunova and Levy, 1999].

Incompatible DNA extremities need to be processed prior to joining. In humans, DNA exonuclease activities were assigned to a complex of DNA-PK and ARTEMIS [Ma *et al.*, 2002], both of which are also involved in the NHEJ-related process of V(D)J recombination [reviewed in: Gellert, 2002; Roth, 2003]. Another complex with 5'-3' exonuclease activity, which therefore was proposed to be involved in end processing, contains the budding yeast proteins Mre11, Rad50 and Xrs2 (MRX complex) [Trujillo and Sung, 2001]. In addition, biochemical studies have shown that MRX can juxtapose DNA ends and stimulate NHEJ [Chen *et al.*, 2001]. When non-complementary ends are joined, synthesis of short DNA stretches is required. This function was assigned to the DNA polymerases Pol4 of yeast [Wilson and Lieber, 1999] and human POLμ [Mahajan *et al.*, 2002]. However, the importance and the exact molecular function of all these end-processing factors in NHEJ are not yet fully understood.

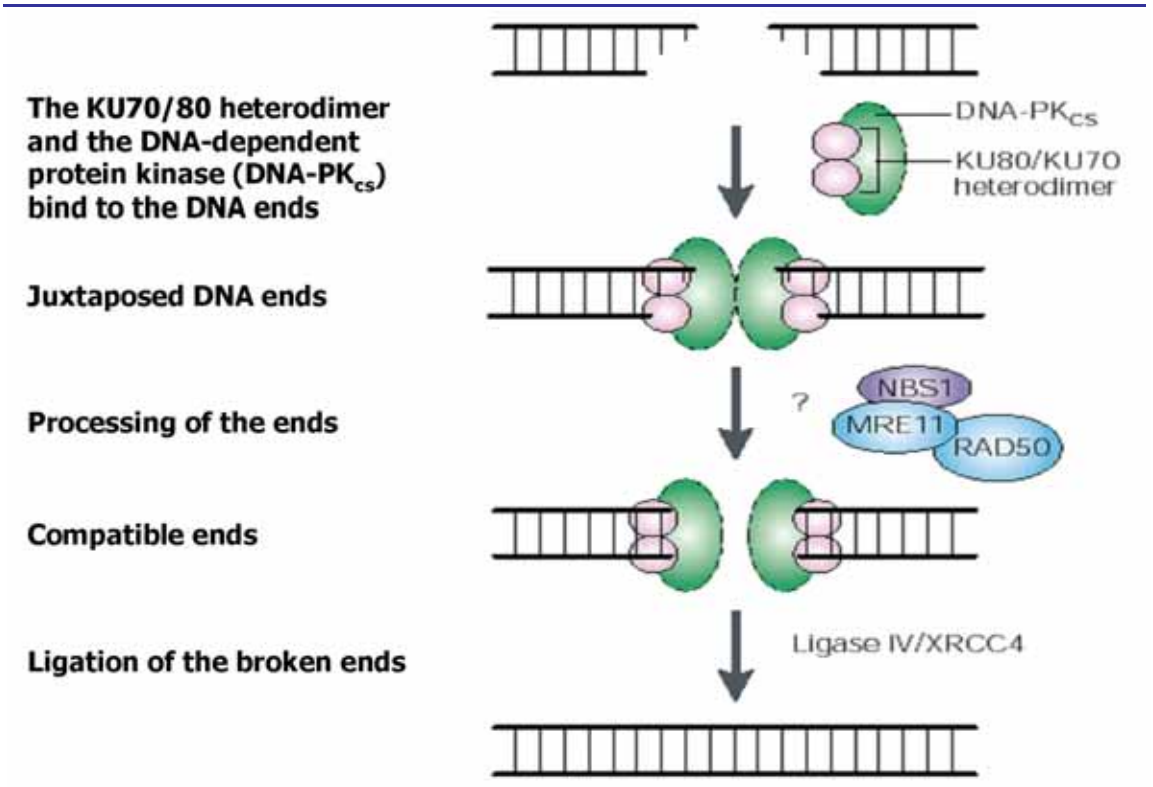


Figure 5: The mechanism of mammalian non-homologous end-joining
The DNA ends are protected, processed and juxtaposed to allow its re-ligation by the DNA ligase IV/ XRCC4 complex. [Adapted from: van Gent *et al.*, 2001].

Once suitable DNA ends are present, they are ligated by the DNA ligase 4 complex: Dln4-Lif1 in yeast [Herrmann *et al.*, 1998; Teo and Jackson, 2000] and Ligase IV/XRCC4 in men [Li *et al.*, 1995; Grawunder *et al.*, 1997; Wilson *et al.*, 1997]. The ligase accessory proteins Lif1 and XRCC4 are proposed to have their function in guiding the ligase complex to the site of juxtaposed DNA ends by its interaction with the Ku complex. The recently described yeast protein Nej1 regulates the subcellular localisation of Dnl4/Lif1 and thereby the capacity to repair DSBs by NHEJ [Valencia *et al.*, 2001]. However, its molecular function remains to be elucidated; it might be a yeast-specific factor since no obvious homologues exist in other organisms (Table 2).

In the last couple of years, NHEJ genes of plants received considerable attention, due to their assumed contribution in the modulation of gene targeting efficiency (Chapter 1.8, page 53). Arabidopsis mutant plants in genes coding for proteins of the Ku, MRX and Lig4 complexes were isolated and described. Their phenotypes basically underlined the presence of the functional conserved NHEJ mechanism (Table 2). Plants mutants in *KU70* and *KU80* were found to be more sensitive to a variety of genotoxic treatments and showed altered telomere lengths [Bundock *et al.*, 2002; Riha *et al.*, 2002; West *et al.*, 2002; Friesner and Britt, 2003; Heacock *et al.*, 2004]. Those phenotypes are in agreement with the findings in yeast and mammalian systems, confirming a central role of the Ku complex in NHEJ and telomere maintenance. Biochemical analysis of the Arabidopsis KU70 and KU80 proteins furthermore confirmed their heterodimerisation and the binding to DNA ends [Tamura *et al.*, 2002]. Interestingly enough, the frequency of repair by HR in a *ku80* background did not change, indicating alternative DSB repair pathways in somatic plant cells [Gallego *et al.*, 2003]. Arabidopsis also possesses a MRE11/RAD50 complex, although the third partner - if it exists - remains to be detected [Daoudal-Cotterell *et al.*, 2002]. Like for the *ku* mutants, telomere length alterations and DNA damage hypersensitivity could be assigned to mutations in *MRE11* [Bundock and Hooykaas, 2002; Heacock *et al.*, 2004] and in *RAD50* [Gallego *et al.*, 2001; Gallego and White, 2001]. In addition, *rad50* mutants revealed an increased frequency of repair by HR in somatic tissue [Gherbi *et al.*, 2001].

In contrast to human, in which mutations in the MRX complex and in the Ligase IV lead to embryonic lethality [Weterings and van Gent, 2004], Arabidopsis plants depleted in all these NHEJ proteins could be obtained, although *mre11* mutants exhibited some growth defects and were sterile [Bundock and Hooykaas, 2002]. Similarly, *rad50* plants were found to be sterile and MMS hypersensitive [Gallego *et al.*,

2001] and had altered telomere length [Gallego and White, 2001]. These sterility phenotypes could be assigned to their function in meiotic HR (see page 48). In contrast, no growth abnormalities but hypersensitivity to DNA damage and full fertility were observed for *lig4* mutants [West *et al.*, 2000; Friesner and Britt, 2003; van Attikum *et al.*, 2003].

All the described analyses of mutants in the NHEJ pathway pointed to the presence of alternative DSB repair pathways in plants. Further support for this hypothesis is provided by cytological analysis of mitotic chromosomes and molecular analysis of telomere patterns in *ku70*, *mre11* and telomerase mutants, singly or in combination [Heacock *et al.*, 2004; Puizina *et al.*, 2004]. Also, the process of *Agrobacterium tumefaciens* T-DNA integration into the genome of plants is thought to depend on NHEJ [Mysore *et al.*, 2000; van Attikum *et al.*, 2001]. Surprisingly, T-DNA integration was not abolished in *ku80* [Gallego *et al.*, 2003] nor in *lig4* mutants [Friesner and Britt, 2003; van Attikum *et al.*, 2003], implying its independence of these NHEJ proteins. The interesting recent description of an Arabidopsis ARTEMIS-like protein SNM1 could be a player in a novel repair pathway; *snm1* plants were shown to be hypersensitive to induction of DSBs and oxidative damages and altered for their repair efficiency [Molinier *et al.*, 2004c]. However, the budding yeast Snm1 was recently shown to be a 5'-exonuclease and proposed to be involved the repair of interstrand crosslinks [Li *et al.*, 2005]; it therefore may have divergent functions in different organisms.

Arabidopsis	Human	Budding yeast	Function
KU70	KU70	yKu70 (Hdf1)	DNA binding, recruitment
KU80	KU80 (KU86)	yKu80 (Hdf2)	DNA binding, recruitment
MRE11	MRE11	Mre11	End processing
RAD50	RAD50	Rad50	End processing
-/?	NBS1	Xrs2	End processing, signalling
XRCC4	XRCC4	Lif1	Juxtaposing of ends, end processing
LIG4	Ligase IV	Dnl4	Ligation
-	-	Nej1	Ligase recruitment
-	DNA-PK_{cs}	-	Signalling, juxtaposing of ends
SNM1?	?	Snm1	End processing
-	ARTEMIS	-	V(D)J
?	POLμ/β?	Pol4	End processing

Table 2: The core proteins of non-homologous end-joining
Described homologues of NHEJ proteins in Arabidopsis, humans and budding yeast and their proposed molecular functions. ?, putative candidate is found in the genome but is not yet described. –, absent homologue.

1.4.3 Mismatch repair

The mismatch repair (MMR) machinery has been mainly implicated in the post-replicative repair of nascent DNA strands, taking care of base-base mismatches and extrahelical nucleotides (loopout) [reviewed in: Kolodner and Marsischky, 1999; Fleck and Nielsen, 2004]. These DNA distortions are caused by misincorporated bases escaping the proofreading activity of polymerases and by polymerase slippage in repetitive sequences. In MMR four main steps can be delineated: A) recognition of the mismatch; B) recruitment of MMR machinery; C) identification of the newly synthesized strand and exonucleolytic degradation beyond the mismatch; and D) resynthesis of DNA (Figure 6) [Hoeijmakers, 2001].

Classical work was done in *E.coli*, resulting in the characterisation of MutL, MutS and MutH as players in the core machinery of MMR. Homologues of MutL and MutS can be found in many eukaryotic organisms (Table 3), indicating a conserved mechanism of MMR [discussed in: Modrich, 1997; Kolodner and Marsischky, 1999; Hays, 2002]. In contrast, homologues of MutH, which is involved in strand discrimination in bacteria, have not been found. This means that this crucial MMR step is performed by a distinct mechanism, adapted to eukaryotic DNA replication.

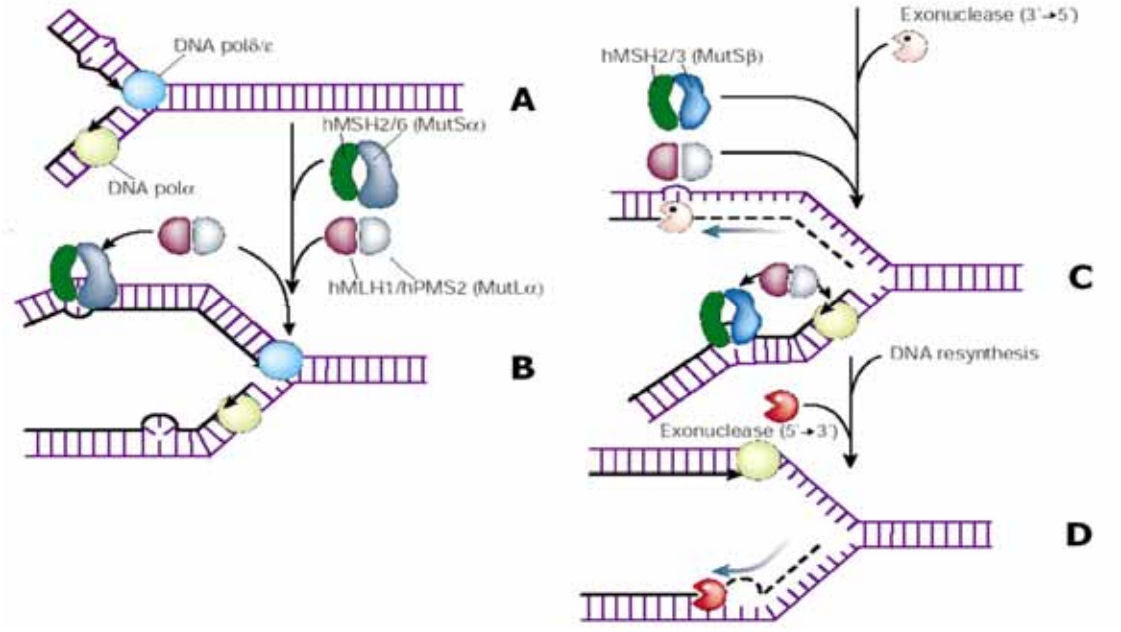


Figure 6: The mechanism of the mammalian mismatch repair
Mismatch repair is tightly connected to DNA replication (**A**). Unfaithful copying of the genetic information is recognised by the MSH2/MSH6 complex (**B**) and by the MSH2/MSH3 (**C**) complexes, which preferentially bind to mismatches or looped structure, respectively. The strand discrimination is mediated by the MLH1/PMS2 complex, presumably by the interaction with the nearby replication machinery. The damage-bearing strand is removed by exonucleases and DNA synthesis restarts (**C, D**). [Adapted from: Hoeijmakers, 2001]

In yeast and humans, heterodimeric complexes with MSH2 recognise post-replicative DNA mismatches [Marsischky *et al.*, 1996; Genschel *et al.*, 1998]. An MSH2-MSH6 complex was reported to detect solely single base mismatches [Greene and Jinks-Robertson, 1997], whereas the MSH2-MSH3 heterodimer also recognises loopout structures [Habraken *et al.*, 1996; Palombo *et al.*, 1996]. Upon damage recognition the heterodimeric MLH1-PMS2 is recruited to the MSH2-containing complex bound to the mispaired bases [Prolla *et al.*, 1994; Li and Modrich, 1995].

MLH1-PMS2 was implied to couple mismatch recognition with the discrimination between template and nascent strand as well as for the recruitment of factors for excision. The mode of eukaryotic strand discrimination is not yet elucidated completely but the polarity of the replication accessory protein PCNA may play a role [Umar *et al.*, 1996; Gu *et al.*, 1998]. Two nucleases were proposed to be involved in the resection of the nascent strand beyond the mispaired bases: EXO1 that interacts physically with MSH2 and FEN1 [Tishkoff *et al.*, 1997a; Tishkoff *et al.*, 1997b; Schmutte *et al.*, 1998]. Finally, the removed bases are resynthesised by the replication DNA polymerases [Tran *et al.*, 1999]. Some of the eukaryotic MMR proteins have, apart from their function in post-replicative repair, reported functions in meiotic recombination or in damage sensing for transcription-coupled repair (see pages 36 and 48).

Arabidopsis	Human	Budding yeast	Function
?	-	Msh1	Mitochondrial MMR
MSH2	MSH2	Msh2	Mismatch recognition
MSH3	MSH3	Msh3	Mismatch recognition, loopout specific
MSH4	MSH4	Msh4	Promoting meiotic HR
?	MSH5	Msh5	Promoting meiotic HR
MSH6	MSH6	Msh6	Mismatch recognition, single base specific
MSH7	MSH7	-	?
MLH1	MLH1	Mlh1	Interface: recognition-strand discrimination
PMS1	PMS2	Pms1	Interface: recognition-strand discrimination
-	PMS1	Mlh2	?
?	-	Mlh3	Interface: recognition-strand discrimination
?	PCNA	PCNA	Strand discrimination
?	EXO1	Exo1	5'-3' exonuclease
?, (osFEN1)	FEN1	Rad27	Structure specific endonuclease
?	POLδ/ε	POLδ/ε	3'-5' exonuclease, DNA synthesis

Table 3: The proteins of the post-replicative mismatch repair machinery
Described homologues of MMR proteins in Arabidopsis, humans and budding yeast and their proposed function. ?, putative candidate is found in the genome but is not yet described. –, absent homologue. os, *Oryza sativa* (rice) homologue.

Little is known about MMR in plants but the presence of Arabidopsis homologues of most of the proteins in the genome propose that this repair pathway is conserved (Table 3) [Culligan and Hays, 1997; Jean *et al.*, 1999; Culligan and Hays, 2000; Hays, 2002]. Of special interest is the existence of the MSH7 protein in higher eukaryotes,

which dimerises with MSH2 and revealed distinct binding preferences to mispaired bases [Wu *et al.*, 2003]. This may reflect the fact that these organisms have to deal with many repeated or homeologous sequences, which request a very tight control of sequence pairing. Indeed, in Arabidopsis *msh2* mutants microsatellite stability was affected [Leonard *et al.*, 2003] and the offspring of these plants accumulated a variety of phenotypic changes, presumably caused by a general genome instability [Hoffman *et al.*, 2004]. Similarly, the over-expression of dominant negative *PMS1* led to an enhancement of microsatellite instability, measured by a synthetic reporter construct [Alou *et al.*, 2004].

1.4.4 Base excision repair

The base excision repair (BER) pathway mainly deals with non-bulky DNA lesions that generally cause minor structural changes of DNA [reviewed in: Nilsen and Krokan, 2001; Drablos *et al.*, 2004]. This basically concerns inappropriate bases in the DNA molecule such uracil, 8-oxo-guanine or thymine glycol, which originate from spontaneous deamination, alkylation and oxidation of bases, respectively. BER was discovered simultaneously in *E.coli* and mammalian cells by Thomas Lindahl and co-workers [Lindahl, 1974; Ljungquist *et al.*, 1974; Ljungquist and Lindahl, 1974]. Presently, the mammalian system is best studied, being divergent from yeast in some aspects [discussed in: Boiteux and Guillet, 2004]. The BER process outlined in Figure 7 involves the removal of the damaged base by substrate specific DNA glycosylases that cleave the glycosylic bond between the base and the deoxyribose. Bifunctional DNA glycosylases with intrinsic lyase activity proceed to incise at the 3' of the abasic (apurinic, AP) site. The remaining AP sites are substrates for the AP endonuclease, which cleaves the 5' phosphodiester bond of DNA strand producing a single-strand break (SSB).

Two independent mechanisms were shown to finalise the repair process by refilling and resealing the gap. The specialised DNA polymerase β (POLβ) with its intrinsic deoxyribophosphodiesterase (dRPase) activity removes the remaining sugar moiety and inserts a matching nucleotide into the gap. Finally, the DNA is sealed by the activity of the human DNA ligase III, mediated by the scaffold protein XRCC1 [Mol *et al.*, 2000; Thompson and West, 2000]. Alternatively, the gap is filled by the DNA polymerase δ/ε complex that synthesises stretches of 2-8 nt, which leads to the displacement of the parental strand. This flap structure is cleaved by the flap endonuclease FEN1 and DNA ligase I is thought to join the ends. Depending on the

length of the synthesised stretches of DNA, these two sub-pathways are termed short patch and long patch BER [reviewed in: Lindahl and Wood, 1999; Krokan *et al.*, 2000].

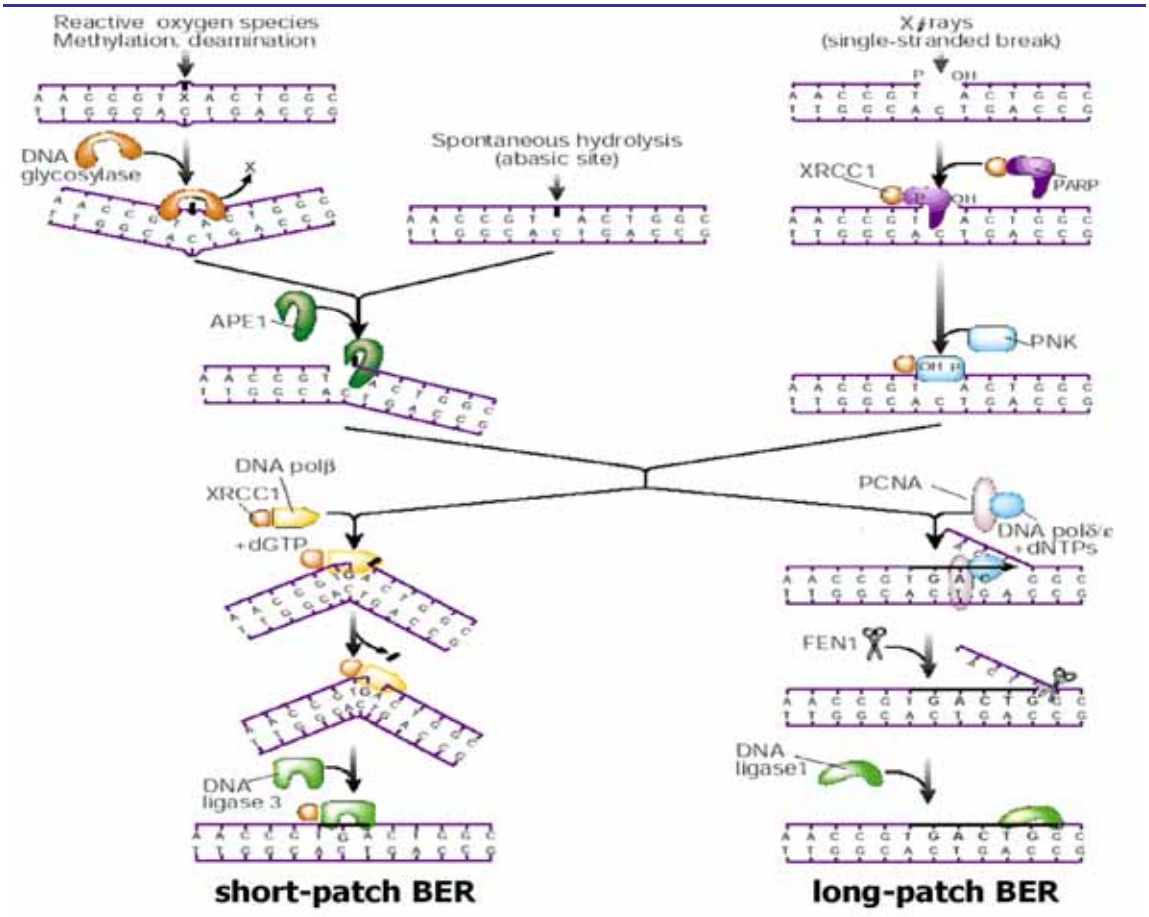


Figure 7: The mammalian base excision repair pathways
Multiple DNA lesions such as base adducts, abasic sites or SSB are processed by a battery of DNA glycosylases and the APE1 endonuclease. Alternatively, some breaks are handled by the XRCC1/Poly(ADP-ribose)-polymerase (PARP) complex and the polynucleotide kinase (PNK). Finally, repair synthesis can be performed by the DNA polymerases β or δ, defining the pathway as short- or long-patch BER, respectively. [Figure taken from: Hoeijmakers, 2001].

The biological significance of the BER pathway and its battery of enzymatic activities vary between organisms. Whereas in budding yeast three types of DNA glycosylases [Boiteux and Guillet, 2004] were described, mammals possess many more, dealing with a wide range of different substrates (Table 4) [Hardeland *et al.*, 2001; Fortini *et al.*, 2003; Hardeland *et al.*, 2003]. BER received a lot of attention because of the frequent C to T point mutations in several types of human cancer, which can arise from the spontaneous hydrolytic deamination of the relatively unstable 5-methyl-cytosine to uracil. The resulting G-U basepair is inevitably mutagenic if replicated prior to recognition by uracil glycosylases and to repair by BER. Despite this proposed function in long-term maintenance of genome integrity, evidence for the involvement of BER in

diseases and cancer proneness as derived from studies with KO mice are rather poor [Fortini *et al.*, 2003]. In fact, no striking phenotypes were observed in animals depleted in any DNA glycosylase, whereas all KOs of genes in later stages of BER were found to be embryo lethal. This suggested an essential role of BER in the post-replicative repair of misincorporated bases (e.g. uracil), which are not recognised by MMR [Nilsen *et al.*, 2000] or in developmental regulation as proposed recently for the differentiation of B cells [Rada *et al.*, 2002]. Coincidently, a mutation in the Arabidopsis DNA glycosylase-related gene *DEMETER* resulted in developmental defects, due to impaired demethylation of an imprinted gene [Choi *et al.*, 2002; Dickinson and Scott, 2002]. Further evidence for a role of DNA glycosylases in the regulation of the DNA methylation was recently provided by the isolation of *ROS1*. Its gene product is a repressor of transcriptional gene silencing by active demethylation of promoters [Gong *et al.*, 2002]. These observations open the field for interesting speculations on the function of BER in the regulation of the state of DNA methylation, of transcription and of epigenetic phenomena in higher eukaryotes. Interestingly enough, an enzymatic DNA demethylation activity could not be isolated so far; this may thus have to happen either passively by DNA replication or indirectly by the action of a repair-related mechanism.

Arabidopsis	Human	Budding yeast	Function
?	UNG1,2	-	DNA Glycosylase
?	SMUG1	-	DNA Glycosylase
?	TDG	-	DNA Glycosylase
?	MBD4	-	DNA Glycosylase
?	UDG2	-	DNA Glycosylase
?	MPG	-	DNA Glycosylase
?	-	Mag1	DNA Glycosylase
OGG1	OGG1,2	Ogg1	DNA Glycosylase/ Lyase
?	MYH	-	DNA Glycosylase/ Lyase
?	NTH1	-	DNA Glycosylase/ Lyase
?	NEIL1,2,3	-	DNA Glycosylase/ Lyase
?	-	Ntg1,2	DNA Glycosylase/ Lyase
MMH	-	-	DNA Glycosylase/ Lyase
?	APE1	Apn1	5' incision
?	-	Apn2	5' incision
os/taPolβ	POLβ	Pol4?	dRPase and DNA synthesis
?	POLδ/ε	POLδ/ε	DNA synthesis
?, osFEN1	FEN-1	Rad27	Excision of 5'dRP
RAD1, UVH1, UVR1	XPF	Rad1	Excision of 3'dRP
ERCC1	ERCC1	Rad10	Excision of 3'dRP

Table 4: The core proteins of the base excision repair machinery
Described homologues of BER proteins in Arabidopsis, humans and budding yeast and their proposed functions. ?, putative candidate is found in the genome but is not yet described. – , absent homologue. os, *Oryza sativa* (rice) homologue. ta, *Triticum aestivum* (wheat) homologue.

Apart from the two reports described above, little is known about BER of plants. Few other DNA glycosylases [Ohtsubo *et al.*, 1998; Dany and Tissier, 2001; Garcia-Ortiz *et al.*, 2001] and rice homologues of FEN1 [Kimura *et al.*, 2001; Kimura *et al.*, 2003] were described molecularly and/or biochemically but a biological significance has not yet been shown (Table 4). In addition, polypeptides were isolated biochemically from rice [Sanathkumar *et al.*, 1996] and from wheat , which resembled the mammalian Polβ in their DNA synthesis characteristics [Luque *et al.*, 1998]. The presence of putative homologues of BER in the Arabidopsis genome suggests the existence and functionality of base excision repair [Hays, 2002].

1.4.5 Nucleotide excision repair

Bulky lesions and adducts which severely distort the helical structure of DNA, are repaired mainly by the nucleotide excision repair (NER) pathway [reviewed in: Petit and Sancar, 1999]. Historically, NER proteins were isolated in screens for UV and IR sensitivity in budding yeast (the Rad genes) and from complementation of human cell lines derived from Xeroderma pigmentosa patients (XP and ERCC genes) (Table 5). People with this inheritable disease exhibit high photosensitivity and skin cancer proneness, indicating the importance of this pathway in UV-damage repair. The NER process consists of five main steps: A) recognition of the damaged DNA; B) remodelling of DNA; C) excision of 24-32 nucleotides proximal to the damage bases; D) filling in of the resulting gap; and E) ligation of the nick (Figure 8) [de Laat *et al.*, 1999; Ura and Hayes, 2002; Costa *et al.*, 2003; Fleck and Nielsen, 2004]. The temporal recruitment and the disassembly of the NER protein were described in a recent publication [Riedl *et al.*, 2003].

Initially, the distorted helical structure of the DNA is sensed by the XPC/hHR23B [Sugasawa *et al.*, 1998; Volker *et al.*, 2001] and the DDB1/DDB2 complexes [Rapic-Otrin *et al.*, 2002]. After the release of the recognition factors the general transcription factor IIH (TFIIH) is recruited to the lesion [Drapkin *et al.*, 1994] and starts to unwind and open the DNA. This is mediated by two of its subunits - XPB and XPD - which have helicase activities of opposite polarity [Schaeffer *et al.*, 1993; Roy *et al.*, 1994]. Subsequently, XPA and the heterotrimeric replication protein A (RPA) bind to the unwound DNA injury and form, together with TFIIH, the so-called preincision complex (Figure 8C).

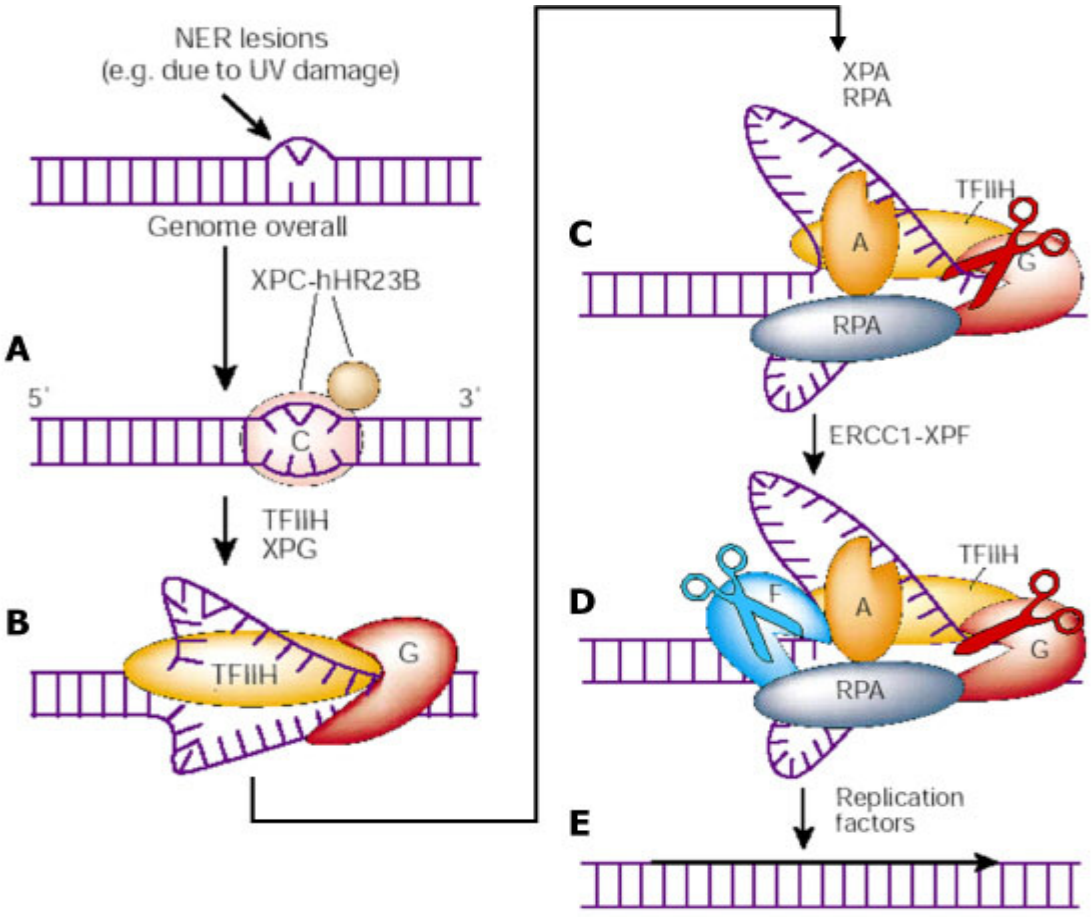


Figure 8: The mechanism of mammalian nucleotide excision repair
A. Damage recognition by XPC-hHR23B. **B.** Recruitment of TFIIH and XPG, opening of the DNA around the lesion. **C.** XPA and RPA stabilise the opened DNA and participate in strand discrimination between damaged and undamaged strand. **D.** ERCC1-XPF are recruited to the repair complex and a DNA stretch of about 24-32 bp around the damage are excised by the endonucleases XPG and XPF. **E.** Repair synthesis and ligation. [Adapted from: Hoeijmakers, 2001].

XPA is anticipated to verify the correct positioning of the repair machinery around the lesion and RPA may preferentially bind to the undamaged DNA in order to confer strand specificity for the incision [de Laat *et al.*, 1998; Dip *et al.*, 2004]. The preincision complex and the ssDNA bubble provides a scaffolding structure for the incoming structure-specific DNA endonucleases XPG and XPF/ERCC1 [Evans *et al.*, 1997a; Evans *et al.*, 1997b]. First, XPG makes the 3' incision on the border of ssDNA and dsDNA [O'Donovan *et al.*, 1994] and the XPF/ERCC1 complex follows with an incision 5' of the injury [Mu *et al.*, 1996; Sijbers *et al.*, 1996]. The DNA polymerases δ and ε were reported to be sufficient for repair synthesis of the created gap, when provided with the replication proteins RPA, PCNA and RF-C [Hunting *et al.*, 1991; Shivji *et al.*, 1995]. The final ligation is performed by the DNA ligase I [Barnes *et al.*, 1992; Prigent *et al.*, 1994].

As expected of a highly conserved repair mechanism, homologues of the main players of NER can be found in plants [Costa, 2001; Hays, 2002]. In an Arabidopsis screen for UV-hypersensitivity (*uvh*), mutants falling into several complementation groups were isolated [Harlow *et al.*, 1994]. Three of them were already shown to be mutated in the Arabidopsis homologues of NER proteins: *UVH1* and *UVH3* are the orthologues of the endonuclease XPF [Fidantsef *et al.*, 2000; Liu *et al.*, 2000] and XPG [Liu *et al.*, 2001], respectively. In an independent study, the Arabidopsis XPF homologue was isolated by a reverse genetic approach and its transcriptional down-regulation resulted in hypersensitivity to various mutagenic treatments [Gallego *et al.*, 2000] and in a reduction of *in vitro* repair activity [Li *et al.*, 2002]. *UVH6* encodes the XPD homologue [Liu *et al.*, 2003], one of the two helicases of the transcription factor TFIIH. One of the two Arabidopsis paralogues of the second helicase XPB was reported to be more sensitive to MMS and to partially complement the yeast *rad25* mutation [Costa *et al.*, 2001].

A genetic screen for γ-radiation hyper-sensitivity yielded the isolation and description of the Arabidopsis *ERCC1* gene [Hefner *et al.*, 2003]. In a screen for somatic hyper-recombination the Arabidopsis *CENTRIN2* gene was isolated; plants mutated in this gene were shown to be impaired in NER and hypersensitive to genotoxic treatment [Molinier *et al.*, 2004a]. Supporting its function in NER, the human homologue of CENTRIN2 stabilises the XPC/hHR23B complex and stimulates NER activity [Araki *et al.*, 2001].

Arabidopsis	Human	Budding yeast	Function
DDB1a, DDB1b	DDB1, XPE	-	
DDB2, DET1	DDB2	-	
-	-	Rad16	Damage binding ATPase
-	-	Rad17	Damage binding
?	XPC	Rad4	Damage sensing, repair recruitment
?	hHR23B	Rad23	Damage sensing, repair recruitment
CENTRIN2	CENTRIN2	-	XPC/hHR23B stabilisation
XPB1	XPB	Rad25/Ssl2	3'-5' helicase
UVH6	XPD	Rad3	5'-3' helicase
?	XPA	Rad14	Complex assembly
UVH3	XPG	Rad2	3' incision
RAD1, UVH1, UVR1	XPF	Rad1	5' incision
ERCC1	ERCC1	Rad10	5' incision
?	RPA (70,32,14)	Rfa(1,2,3)	Complex stabilisation
?	POLδ/ε	POLδ/ε	DNA synthesis

Table 5: The core proteins of nucleotide excision repair machinery
Described homologues of NER proteins in Arabidopsis, humans and budding yeast and their proposed functions. ?, putative candidate is found in the genome but is not yet described. –, absent homologue.

1.4.6 Other repair activities

1.4.6.1 Reversal of *O*-alkylated bases by alkyltransferases

Alkyl groups at the O^6-position of guanine are highly mutagenic [Drablos *et al.*, 2004]. Most organisms possess alkyltransferases, which are able to catalyse the irreversible transfer of the mutagenic alkyl (mostly methyl) group to a cysteine residue. Such enzymes exist in bacteria [Sedgwick and Lindahl, 2002], lower eukaryotes [Xiao *et al.*, 1991] and mammals [Pegg, 2000] but not in plants [Hays, 2002].

1.4.6.2 Photoreactivation

Exposure to UV light induces two major photoproducts in DNA: cyclobutane pyrimidine dimers and pyrimidine-(6-4)-pyrimidinones (Chapter 1.3.2, page 8). Photolyases belong to a protein class, which are specific for a particular type of DNA photoproducts and can enzymatically reverse the modified base in a light-dependent reaction [Sancar, 1996; Deisenhofer, 2000]. These enzymes are considered an efficient backup system for the repair of UV-induced damages generally repaired by NER. In most organisms but not in humans at least one type of photolyase was isolated [discussed in: Thoma, 1999; Hays, 2002]. A series of Arabidopsis mutants deficient in the repair of these DNA damages was isolated in a genetic screen for UV-B sensitivity (*uvr*) [Jiang *et al.*, 1997]. *Uvr2* was impaired in the light-dependent repair of CPDs but not of 6-4PPs [Landry *et al.*, 1997]; the mutated gene corresponded to *PHR1*, identified to code for a plant type II photolyase [Ahmad *et al.*, 1997]. An Arabidopsis photolyase (UVR3) specific for 6-4PP was also isolated in the same screen [Nakajima *et al.*, 1998]. The presence of at least two photolyases in Arabidopsis and an UV-absorbing pigment shield is apparently required to protect the plant genome from exposure to UV-B irradiation of solar light.

1.4.6.3 UV-damaged DNA endonuclease-dependent excision repair

This repair pathway (UVER) was only described in the fission yeast *Schizosaccharomyces pombe*, an organism lacking photolyase activities, and in *Neurospora crassa* as well [Freyer *et al.*, 1995; McCready *et al.*, 2000]. The UV-damage DNA endonuclease (UVDE) recognises CPD and 6-4PP photoproducts and induces a nick 5' of the lesion. This incision was anticipated to be the substrate for BER-like process, since UVER was found to depend on the presence of the *S.pombe* homologue of FEN1 [Yonemasu *et al.*, 1997].

1.4.6.4 Nucleotide incision repair

Nucleotide incision repair (NIR) was discovered only recently as an alternative repair activity for oxidative damage [Ischenko and Saparbaev, 2002]. Its discovery had been stimulated by the observations that DNA glycosylase-deficient mice and *E.coli* were still able to remove oxidised bases [Gros *et al.*, 2002]. NIR resembles the BER and the *S.pombe* UVER pathways, initiating a single incision 5' to the oxidised base that acts as a starting point for repair synthesis of DNA stretches. The resulting flap structure is processed by FEN-1 or by an alternative structure-specific endonuclease. Unlike BER, NIR does not depend on the action of DNA glycosylases to produce AP sites. The major human AP endonuclease (APE1) was recently found to be involved in NIR, incising the DNA 5' of oxidative damage without prior removal of the damaged base [Gros *et al.*, 2004]. The existence of this repair pathway in plants remains to be analysed.

1.5 DNA damage surveillance mechanisms

Damage sensory proteins are constantly scanning the genome for distorted DNA structure and aberrant base composition [reviewed in: Cline and Hanawalt, 2003]. These proteins are able to recognise and to specifically bind to all sorts of damages. Either they immediately reverse the damage by their intrinsic enzymatic activity or they trigger the recruitment of other repair factors. Alternatively, DNA damages are detected by cellular processes such as transcription and replication (see below). Although these DNA lesions are repaired by many distinct mechanisms with their specific sets of proteins (see above), there are common signal transduction pathways, which result in the slowing down or arrest of cell cycle progression [Rouse and Jackson, 2002]. These control mechanisms for genome integrity and repair are generally termed DNA damage "checkpoints" and act in all stages of the cell cycle of eukaryotic organisms. They orchestrate the sensing of lesions, the recruitment and the activity of repair proteins, they control the progression of DNA replication and they signal to cell cycle-regulating molecular pathways [reviewed in: O'Connell *et al.*, 2000; Kolodner *et al.*, 2002; Furuya and Carr, 2003; Sancar *et al.*, 2004]. The detection and the repair of DNA damage in the tightly packed chromatin increase the level of genome maintenance complexity and emphasise the role of chromatin remodelling activities in efficient and accurate removal of DNA lesions. Accessibility of repair proteins to sites of DNA damage may require changes of chromatin structure or temporal removal of nucleosomes, which need to be reset after successful repair [reviewed in: Ehrenhofer-Murray, 2004; Koundrioukoff *et al.*, 2004; Peterson and Cote, 2004].

1.5.1 Cellular responses to DNA double-strand breaks

DSBs belong to the DNA damages the most hazardous and most difficult to repair. Failure or unfaithful repair of them may result in chromosomal instability especially harmful during progression through S- and in M-phase of the cell cycle. Eukaryotes have evolved a variety of cellular machineries, which act in a concerted fashion to prevent the deleterious effects of a DSB. In yeast, even a single break can rapidly be detected and can trigger a global DNA damage response [Bennett *et al.*, 1993; Lee *et al.*, 1998]. In yeast and mammalian cells, the aggregation of multiple proteins to cytological foci was observed at sites of DSBs. They are marked by specific chromatin structures and the presence of repair and checkpoint proteins [reviewed in: Lisby and Rothstein, 2004]. These foci are thought to be “repair centres”, allowing the coordinated and simultaneous repair of several DSBs [Lisby *et al.*, 2003; Aten *et al.*, 2004]. Such repair centres were reported at DSBs and SSBs, at sites of replication stress, at shortened telomeres or at chromosomal fragments in senescing cells [Raderschall *et al.*, 1999; Ward and Chen, 2001; d'Adda di Fagagna *et al.*, 2003; Lisby *et al.*, 2003; Sedelnikova *et al.*, 2004].

The human MRE11/RAD50/NBS1 complex (MRN) and the human protein kinase ataxia telangiectasia-mutated (ATM) play a central role in the cellular response to DSBs [Lavin, 2004]. The assembly of the repair centres is initiated by the detection of DSBs most probably by the human MRN complex, which is quickly recruited to the damaged sites [Mirzoeva and Petrini, 2001; discussed in: Petrini and Stracker, 2003]. The homologous yeast complex MRX may also be involved in the temporary stabilisation of the DSB ends, which allows the repair by the NHEJ pathway [Frank-Vaillant and Marcand, 2002]. In fact, MRE11 is required for the activation of the central transducer of DSB response ATM, supporting a sensory role of the MRN complex [Carson *et al.*, 2003; Uziel *et al.*, 2003]. Upon induction of DSBs, the yeast homologues of MRE11 and ATM are quickly recruited to sites of damage [Lisby *et al.*, 2004]. Subsequently, ATM is converted to its active monomeric form by the MRN complex and by autophosphorylation [Bakkenist and Kastan, 2003; Lee and Paull, 2004] and localises to DNA ends [Smith *et al.*, 1999; Andegeko *et al.*, 2001]. Activated ATM phosphorylates many downstream proteins, which facilitate the assembly of the repair centres, the arrest of cell cycle progression and the repair of the DSB (e.g.: the tumour suppressors p53, BRCA1 and CHK1) [reviewed in: Abraham, 2001; Sancar *et al.*, 2004]. Consistent with its proposed role in orchestrating DSB repair, Arabidopsis plants mutated in the *ATM* gene exhibited meiotic defects, enhanced sensitivity to DSB

induction and failed to induce transcription of repair genes upon DNA insults [Garcia *et al.*, 2000; Garcia *et al.*, 2003].

The rapid phosphorylation of the mammalian histone variant H2AX (designated as γ-H2AX) at DSBs by ATM [Burma *et al.*, 2001] underscores the importance of chromatin structure in the assembly of the repair machinery [Rogakou *et al.*, 1998; Rogakou *et al.*, 1999]. H2AX depleted mice were found to be radiation sensitive and there was an alteration in the recruitment and persistence of regulatory proteins such as BRCA1 and NBS1 to DSBs. Interestingly enough, damage recognition and recruitment of repair factors (such as RAD51) were not impaired, supporting the suggestion that these chromatin marks take part in the assembly of repair centres and in regulatory mechanisms of repair [Celeste *et al.*, 2002; Celeste *et al.*, 2003]. The budding yeast functional homologue of γ-H2AX - serine 129 phosphorylated H2A - is required for the recruitment and binding of the chromatin remodelling complex INO80 to sites of DSBs [Morrison *et al.*, 2004; van Attikum *et al.*, 2004]. Depletion of INO80 complex subunits rendered yeast cells sensitive to DNA damaging agents, presumably because of impaired repair by both HR and NHEJ [Shen *et al.*, 2000; van Attikum *et al.*, 2004]. In agreement with these observations, a mutation in the orthologous Arabidopsis gene *INO80* significantly suppressed the use of HR to repair DSBs [Fritsch *et al.*, 2004]. The INO80 complex as well as other chromatin remodelling and histone modifying complexes may be involved in the displacement of histones at sites of DSB, allowing the resection of DNA ends and facilitating repair by HR [discussed in: Cairns, 2004].

1.5.2 Control of repair

Whereas ATM is mainly involved in DNA DSB signalling to downstream targets of the cascade, the human “ATM and spRad3-related” (ATR) protein kinase is proposed to play a more general role in the surveillance of repair and replication activities. This notion is supported by several lines of evidence: 1) Only ATR is activated upon exposure to UV or treatment with genotoxic agents that produce bulky lesions [Abraham, 2001; Cortez *et al.*, 2001]. 2) ATR binds directly to UV-induced lesions whereas its recruitment to DSBs depends on RPA covering the resected ssDNA [Ünsal-Kaçmaz *et al.*, 2002; Zou and Elledge, 2003]. 3) Mice knocked-out for *ATM* were viable but prone to cancer, retarded in growth and exhibited meiotic chromosome fragmentation and immune deficiencies [Xu *et al.*, 1996]. In contrast, depletion of ATR in mice or even in human cell lines resulted in chromosome breaks and cell death,

most likely due to its checkpoint function in DNA replication [Brown and Baltimore, 2000; de Klein *et al.*, 2000; Cortez *et al.*, 2001]. The situation in plants differs significantly from that of mammals; Arabidopsis plants depleted of ATR were found to be viable and phenotypically normal [Culligan *et al.*, 2004]. However, a comparable function in checkpoint control was underscored by the failure of induced G2 cell cycle arrest and by the hypersensitivity of *atm* plants to treatments which led to replication stalling or bulky DNA lesions but not to the induction of DSBs (Table 6).

Arabidopsis	Human	Budding yeast	Function
MRE11/RAD50/?	MRE11/RAD50/NBS1	Mre11/Rad50/Xrs2	DSB sensing
?	RPA	RFA	Damage sensing
ATM	ATM	Tel1	Checkpoint protein
ATR	ATR	Mec1	Checkpoint protein
RAD17-RFC?	RAD17-RFC	Rad24-RFC	Checkpoint complex
RAD9/?/?	RAD9/HUS1/RAD1	Ddc1/Mec3/Rad17	Checkpoint complex

Table 6: The checkpoint-mediating proteins
Described homologues of DNA damage checkpoint proteins of Arabidopsis, humans and budding yeast and their proposed functions. ?, putative candidate is found in the genome but is not yet described.

The two major human checkpoint-mediating protein kinases ATR and ATM appear to signal certain types of DNA lesions. They have partially redundant functions and share many down-stream targets, which provoke similar cellular responses [discussed in: Abraham, 2001; Sancar *et al.*, 2004]. Apart from ATM or ATR, a functional checkpoint response in human depends on two other protein complexes: RAD17-RFC and RAD9/RAD1/HUS1 (9-1-1) [discussed in: Melo and Toczyski, 2002]. In the RAD17-RFC complex the biggest subunit of RFC is replaced by the checkpoint-specific RAD17 protein [Lindsey-Boltz *et al.*, 2001]. These two complexes are considered structural homologues of the replication factor C (RFC) and proliferation cell nuclear antigen (PCNA) (see page 43) and are thought to perform analogous functions in checkpoint response. As for ATR the recruitment of RAD17-RFC onto damaged DNA depends on RPA, underlining the crucial function of RPA in DNA damage sensing and induction of checkpoint response [Zou *et al.*, 2003]. Subsequently, the RAD17-RFC complex loads the 9-1-1 complex onto DNA in an ATP-dependent manner [Lindsey-Boltz *et al.*, 2001; Bermudez *et al.*, 2003; Majka and Burgers, 2003]. The functional conservation of the checkpoint-mediating complexes in Arabidopsis was underlined by the analysis of *rad17* and *rad9* mutant plants which both revealed enhanced sensitivities to genotoxic treatments and genome instability in an epistatic manner [Heitzeberg *et al.*, 2004].

1.5.3 Genome surveillance by the transcription machinery

Eukaryotic transcription is carried out by the RNA polymerase holoenzymes (RNAP) I, II and III, which are responsible for the production of rRNA, mRNA and snRNA, and tRNA , respectively [Lodish, 2000]. The initiation of transcription and the elongation of the RNA strand are mediated by many proteins and protein complexes such as general transcription factors and are tightly regulated by the orchestrated assembly and disassembly of them. Transcription initiation requires unphosphorylated RNAPIIs and the switch to the elongation phase is marked by hyperphosphorylation of its largest subunit [Lee and Young, 2000; Sims *et al.*, 2004]. Remarkably, one of these transcription factors (TFIIH) is one of the key players in the NER as well as in the BER pathway (see page 28) [Le Page *et al.*, 2000a]. This suggests a close relationship between transcription and repair mechanisms, termed transcription-coupled repair (TCR) [Zurita and Merino, 2003].

The first hint about a link of transcription and repair was provided by the findings in human cells that active genes are faster repaired than inactive ones but only the transcribed strands of them [Mellon *et al.*, 1986; Mellon *et al.*, 1987]. Originally, TCR was described as a sub-pathway of NER but recently it became clear that also DNA lesions removed by BER are repaired in a transcription-dependent fashion [Cooper *et al.*, 1997; Le Page *et al.*, 2000a]. Therefore, TCR is more generally defined as repair activity, triggered by DNA damage on the transcribed strand, which leads to stalling of the RNAP transcription machinery [reviewed in: Svejstrup, 2002]. Interestingly enough, a role in the TCR induction of certain oxidative lesions was assigned to the MMR proteins MLH1 and MSH2 (Figure 9A). It was proposed that they are able to detect and to bind tightly to these potentially mutagenic bases, leading to the blockage of the RNAP progression and thereby inducing TCR [Duckett *et al.*, 1996; Hickman and Samson, 1999]. Moreover, at least in human cells the DNA damage sensing capacity of transcription was proposed to have an essential role in the maintenance of genome integrity. Above a certain threshold of stalled RNAPs and reduction of the transcription level apoptotic mechanisms are induced in a p53-dependent manner [Ljungman and Lane, 2004].

1.5.4 Transcription-coupled repair

A central role in TCR was assigned to the Cockayne Syndrome group B (CSB) protein, which is the human homologue of the *S.cerevisiae* Rad26. Patients with this syndrome reveal severe phenotypes such as dwarfism, neurological abnormalities, premature

aging and photosensitivity [Nance and Berry, 1992]. CSB interacts with a series of proteins, which are involved either in transcription or in DNA repair [Licht *et al.*, 2003]. The protein was found in the RNAPI and II complexes [Tantin *et al.*, 1997; van Gool *et al.*, 1997; Bradsher *et al.*, 2002], where it interacts with several subunits of TFHII and other general transcription factors [Selby and Sancar, 1997b; Tantin, 1998]. Furthermore, interactions of CSB were reported with the NER proteins XPG and XPA as well as with its binding partner XAB2 [Iyer *et al.*, 1996; Selby and Sancar, 1997b; Nakatsu *et al.*, 2000] and with the DNA glycosylase OGG1 [Tuo *et al.*, 2002]. The CSB protein belongs to the SNF2 family of DNA-dependent ATPases, containing the seven consecutive ATPase domains [Eisen *et al.*, 1995]. In fact, CSB was shown to hydrolyse ATP in a DNA-dependent manner, to interact with the tails of all four core histones and to have chromatin remodelling activities [Selby and Sancar, 1997b; Citterio *et al.*, 2000]. Moreover, a bubble-structured DNA resembling opened DNA in NER or in transcription stimulated the ATPase activity of CSB whereas damaged DNA did not, excluding a role in damage sensing [Christiansen *et al.*, 2003].

CSB was also reported to interact with and to stimulate the hyperphosphorylated RNAPII-elongation complex, which suggests a role as elongation factor [Selby and Sancar, 1997a]. In concordance to this, *csb* cells revealed a reduced level of general transcription [Balajee *et al.*, 1997]. Upon treatment with agents inducing TCR such as UV-irradiation the transcriptional activity is substantially reduced in both wild-type and *csb* cells, presumably to avoid the effects of transcriptional mutagenesis. This reduction is mediated either directly by stalling RNAPs or indirectly by inducing cellular DNA damage response pathways and the depletion of unphosphorylated RNAPs and thus a decrease in the rate of transcription initiation [reviewed in: Svejstrup, 2002]. Interestingly enough, the tumour suppressors BRCA1 and BRCA2 were found to be required for TCR [Le Page *et al.*, 2000b]. BRCA1 was found to be associated with the elongating RNAPII complex, whereby it becomes phosphorylated (probably by ATM or ATR). Upon DNA insult and RPAPII stalling it is released, indicating an important function of BRCA1 in DNA damage signalling following transcriptional block [Krum *et al.*, 2003]. However, in wt cells a considerable proportion of the RNAPII complexes becomes ubiquitylated and degraded before RNA synthesis is resumed. These processes were reported to be very inefficient in *csb* cells, as well as in CS group A cells (CSA), causing apoptosis and subsequently the CS phenotype [Luo *et al.*, 2001; McKay *et al.*, 2001]. Human cells mutated in *CSA* or *CSB* are also deficient in transcription-coupled repair of DNA [Troelstra *et al.*, 1992; Henning *et al.*, 1995]. In

conclusion, CSB may have a critical gatekeeper function in the switch between repair and transcription mode, most probably allowing the access of repair proteins to obstructing lesions at stalled RNAPII sites. The molecular mechanism of CSB action, as well as its interplay with other TCR proteins, is controversial and heavily debated, and several models were proposed (Figure 9) [reviewed in: Svejstrup, 2002; Ljungman and Lane, 2004].

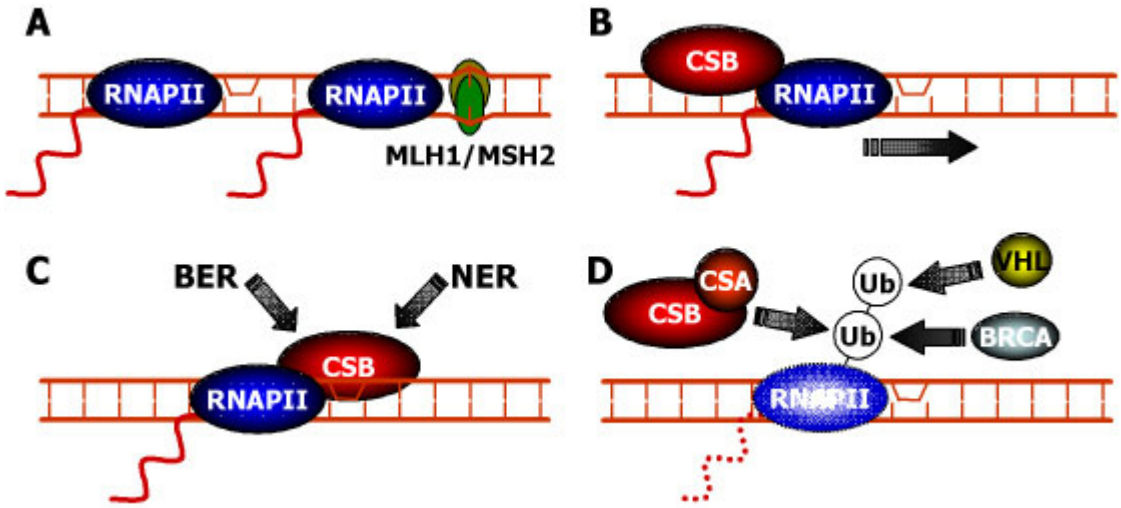


Figure 9: Tentative models for CSB function in transcription-coupled repair
A. The RNA polymerase II (RNAPII) can stall at bulky DNA lesions or at base lesions, which are marked by the tightly bound mismatch repair proteins MLH1/MSH2. **B.** CSB is mediating translesion synthesis across the DNA lesion by pushing forward the stalled RNAPII. **C.** CSB recruits BER and NER factors, which remove the DNA lesion, allowing RNA synthesis to proceed. **D.** CSB as well as other proteins (CSA, BRCA1, VHL) mediate the ubiquitylation of the RNAPII and its subsequent degradation by the proteasome, which enables the repair of a damage and resuming of RNA synthesis.

A first model suggests that CSB promotes lesion bypassing of RNAPs either by its intrinsic ATPase activity or by the recruitment of accessory factors (Figure 9B). Supports for this hypothesis are provided by the ability of CSB to stimulate stalled RNAPIIs to extend the nascent RNA by an additional nucleotide [Selby and Sancar, 1997a] and by a recent report from the putative prokaryotic CSB homologue, which was shown to push the RNAPs over the DNA lesion in an ATP-dependent manner [Park *et al.*, 2002]. Alternatively, the chromatin remodelling activity of CSB may facilitate transcription elongation as well as change the RNAPII-DNA interface in a way that allows the recruitment and binding of repair factors (Figure 9C). This model is supported by the fact that the initiator of NER - XPC - is not required for TCR [Mu and Sancar, 1997]. A further model for the TCR proposes that CSB promotes the removal of stalled RNAPII by ubiquitin-mediated proteolysis, which was already previously

implied in transcription regulation (Figure 9D) [reviewed in: Muratani and Tansey, 2003].

Evidence for such a model was recently provided by the discovery of a new player in TCR - Def1 - in budding yeast [Woudstra *et al.*, 2002]. Def1 was found in a complex with Rad26, coordinating the proteolytic degradation of the stalled RNAPII complex by ubiquitylation [van den Boom *et al.*, 2002] . Although homologues for Def1 could not be identified in higher eukaryotes (Table 7), there are several protein complexes with ubiquitin-ligase activity reported in the TCR context, which could mediate the proteolytic removal of RNAPII [Ljungman and Lane, 2004]. Firstly, a complex containing CSA as well as the NER protein DDB2 was reported to have DNA damage-regulated ubiquitin-ligase activity [Groisman *et al.*, 2003]. Secondly, the tumour suppressor protein von Hippel-Lindau (VHL) bound to elongating RNAPII upon UV-irradiation and targeted it for ubiquitylation [Kuznetsova *et al.*, 2003]. Thirdly, a complex containing BRCA1 exhibited ubiquitin-ligase activity [Krum *et al.*, 2003].

The process of TCR might be conserved from bacteria to man, although some substantial differences were found (see above). It is therefore very likely that plants also possess a similar system for their genome surveillance as indicated by the presence of putative orthologues (Table 7) [Hays, 2002]. However, apart from reports on BRCA1 [Lafarge and Montane, 2003] and UVH3 (the hXPG/yRad2 homologue) [Liu *et al.*, 2001], little is known about transcription-coupled repair of plants. Strikingly, upon induction of oxidative damage *uvh3* Arabidopsis mutants exhibited a premature senescence phenotype comparable to *csb* cells, underlining the functional conservation of TCR in plants.

Arabidopsis	Human	Budding yeast	Function in TCR
?	CSB	Rad26	Mediator of TCR, transcription elongation
?	CSA	Rad28	RNAPII degradation
-	-	Def1	RNAPII degradation
DDB2	DDB2	-	Damage sensing?, RNAPII degradation
?	TFIIH	TFIIH	Transcription initiation, TCR
?	XPA	Rad14	Assembly of the repairosome?
?	XAB2	Syf1	Transcription recovery
UVH3	XPG	Rad2	Endonuclease for NER or BER
BRCA1	BRCA1	-	RNAPII degradation, damage signalling
BRCA2	BRCA2	-	?
?	VHL	-	RNAPII degradation

Table 7: Proteins proposed to be involved in transcription-coupled repair
Described homologues of TCR-mediating proteins in Arabidopsis, humans and budding yeast and their proposed functions. ?, putative candidate is found in the genome but is not yet described.

1.6 DNA replication

The genome duplication during the S-phase of the cell cycle is a key step of cell proliferation, bearing a high mutagenic risk. Therefore, it is intimately controlled by the concerted action of DNA integrity checkpoints, cell cycle regulators and the replication machinery. Previously unrepaired DNA damages and incomplete, superfluous or inaccurate DNA synthesis would lead to genome alterations, which are putatively deleterious to the descending cell generation. However, it was estimated that one round of replication in human cells yields less than one base change, which is astonishingly low in respect of possible obstacles and problems the replication machinery could face [Drake *et al.*, 1998].

Key steps of replication such as the initiation, regulation and assembly of the core DNA synthesis factory (replisome, synthesome) are highly conserved within eukaryotes and to some extent also in bacteria [discussed in: Gilbert, 2001; Bell and Dutta, 2002; Kearsey and Cotterill, 2003]. Variations were mainly reported for sites of replication initiation and for protein complexes with control functions, which presumably evolved as consequence of the large genome sizes of mammals or plants. Eukaryotic DNA replication is initiated at discrete positions called replication origins which are spread throughout the whole genome. Replication forks are established at origins and DNA synthesis starts bidirectionally, forming an expanding replication bubble (Figure 10 and Figure 11). DNA synthesis and fork migration continues until it joins a replication bubble, which was fired at a neighbouring origin. During replication not only the DNA sequence is copied but also epigenetic marks and the higher order chromatin structure. Sophisticated mechanisms are needed for an accurate and fast replication through the inhibitory chromatin and the subsequent re-establishment of chromatin structure and epigenetic information [discussed in: McNairn and Gilbert, 2003; Ehrenhofer-Murray, 2004].

1.6.1 Replication initiation

Late in the G1-phase several protein complexes sequentially assemble on the chromatin and form the pre-replication complex (preRC) (Figure 10). In *S.cerevisiae* the replication origins are defined by short sequences (autonomously replicating sequences: ARS) [Rao and Stillman, 1995], to which the multiproteinous origin recognition complex (ORC) binds in an ATP-dependent manner [Klemm and Bell, 2001]. In other species, the sequence specificity of ORC binding differs from the situation in budding yeast [discussed in: Gilbert, 2001]. For instance, human ORC

binds to virtually any DNA sequence and initiates replication *in vitro*, although replication origins were mapped into distinct regions [Ladenburger *et al.*, 2002; Vashee *et al.*, 2003]. The position of preRC assembly may be controlled by other mechanisms such as chromatin structure or by preferential binding of ORC proteins to AT-rich sequences as reported for *S.pombe* [Kong *et al.*, 2003; Dai *et al.*, 2005].

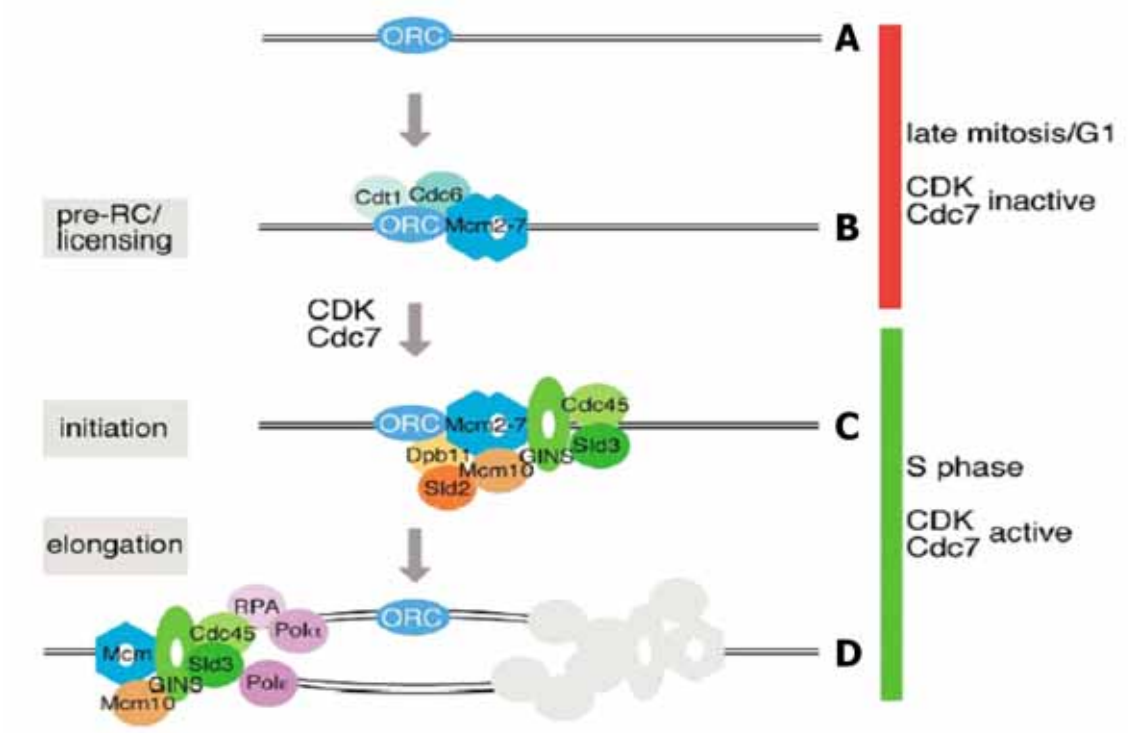


Figure 10: The initiation of DNA replication
The initiation and the transition to DNA replication involves a complex molecular network of protein loading and activation which is concerted by cell cycle-regulating cascades. **A.** In the G1-phase the ORC complex binds to the origin of replication. **B.** Mediated by Cdt1 and Cdc6, the MCM complex is recruited and loaded onto the ORC complex, forming the pre-replication complex (pre-RC). **C.** Upon entry into the S-phase, Cdc45 promotes DNA unwinding by MCM and the loading of many accessory proteins onto the pre-RC. **D.** Finally, RPA, Polα and Polε are recruited to the fork and initiate DNA synthesis, resulting in the assembly of the replication fork with Polδ (Figure 11). The replication bubble migrates bidirectionally away from the origin of replication. [This illustration is taken from: Kearsey and Cotterill, 2003].

Subsequently, yeast Cdc6 interacts with ORC proteins in the preRC; the assembled complex structurally resembles the replication clamp loader RF-C (see below) and may facilitate the loading of the MCM complex (mini chromosome maintenance proteins) onto the preRC in a similar way [Perkins and Diffley, 1998]. Cdt1 controls and promotes the loading of the MCM complex onto chromatin, ensuring correct timing of replication initiation and preventing re-replication. In yeast, Cdt1 is localised to the nucleus only for a short time window during the late G1-phase [Tanaka and Diffley,

2002]. In mammalian systems there is an additional level of regulation: MCM loading activity of human CDT1 is negatively regulated by its interaction with geminin [Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001]. The MCM complex consists of six subunits (Mcm2-7) which are highly related to each other on the sequence level. It is required for replication licensing [Prokhorova and Blow, 2000] and also for fork progression in S-phase [Labib *et al.*, 2000].

Recently, biochemical evidence suggested that the MCM complex may act as a replicative DNA helicase in yeast [Aparicio *et al.*, 1997; Labib and Diffley, 2001]. In contrast, higher eukaryotes encode an MCM-related protein - MCM8 - which was proposed to function as replicative DNA helicase [Maiorano *et al.*, 2005]. In addition to the above presented control mechanisms, the assembly of the preRC is tightly regulated by the cyclin-dependent kinases (CDK), which were shown to phosphorylate Cdc6 and proteins of the ORC and the MCM complex [reviewed in: Jallepalli and Kelly, 1997; Bell and Dutta, 2002].

1.6.2 Transition to DNA replication

The entry into the S-phase is associated with high activities of the cyclin-dependent kinases and the yeast Cdc7 protein [reviewed in: Masai and Arai, 2002]. Targets of these two protein kinases are components of the preRC as well as proteins that enable the transition to DNA synthesis; their activities are greatly influenced by the phosphorylation status. The transition of the preRC into the synthesome consists of two major steps: the unwinding of the DNA at licensed replication origins and the recruitment of DNA polymerases and their accessory proteins (Figure 10C, D). Several protein complexes are involved in this critical step in a mutually interdependent fashion, some of them remaining associated as integral components of the processive replication fork [reviewed in: Bell and Dutta, 2002]. Cdc45 emerges as a pivotal factor of the S-phase transition; upon activation by CDKs and Cdc7, it binds to chromatin and stimulates the MCM complex to unwind the origins. The unwound ssDNA then promotes the recruitment of the yeast replication factor A (RFA; SSB or RPA in other organisms) [Zou and Stillman, 1998; 2000] and the Dpb11-mediated loading of the DNA polymerases α and ε [Masumoto *et al.*, 2000]. Other factors are found to participate in the transition to DNA replication but their exact molecular functions remain to be elucidated. Sld3 binds to Cdc45 and is - like the GINS complex - required for the assembly of the replication fork [Kamimura *et al.*, 2001; Takayama *et al.*, 2003]. Mcm10 was reported to be essential for an efficient transition to replication by

its interaction with the MCM complex and for replication fork progression [Homesley *et al.*, 2000; Kawasaki *et al.*, 2000]. Interestingly enough, the loading of the DNA polymerases and accessory proteins onto the replication fork appears to be sequential, ensuring the presence of the complete replisome when DNA synthesis starts. Firstly, the DNA polymerase ε (Polε) complex is recruited to origins mediated by its subunit Dpb11 (see above), independent of polymerase α (Polα) or RPA loading [Masumoto *et al.*, 2000]. In contrast, the assembly of the Polα complex requires the presence of Polε and of ssDNA coated by RPA at the unwound origins. This leads to the synthesis of *bona fide* replication primers, a prerequisite for the loading of the processivity factor of DNA synthesis, the proliferating cell nuclear antigen (PCNA) and subsequently of polymerase δ (see below) [Mimura *et al.*, 2000]. Interestingly enough, apart from its crucial role in replication initiation, the heterotrimeric RPA has proposed functions in DNA repair and signalling [reviewed in: Wold, 1997].

1.6.3 The replication machinery and DNA synthesis

The processive replication fork consists of the three essential DNA polymerase holoenzymes α, δ and ε and a series of accessory proteins, which are required for the rapid and errorless duplication of a whole genome [reviewed in: Waga and Stillman, 1998]. Due to the 5'-3' polarity of all DNA polymerases [Hübscher *et al.*, 2002], DNA synthesis on the leading strand is continuous whereas on the lagging strand discrete stretches of about 100 to 200 bp (Okazaki fragments) are synthesised. These have to be processed and rejoined (Okazaki fragment maturation, see below) [Kao and Bambara, 2003].

DNA replication of both leading and lagging strand is initiated by the DNA polymerase α-primase holoenzyme [Waga and Stillman, 1994], which consists of four essential subunits in all organisms [Hübscher *et al.*, 2002]. Among the DNA polymerases, Polα has unique features; it can initiate DNA synthesis *de novo* - without the need of primers - by first synthesising about 10 nt RNA followed by 30 nt of DNA [Waga *et al.*, 1994]. This short RNA-DNA hybrid serves as a starting site for DNA extension by the more processive DNA polymerases δ (Polδ) or ε (Figure 11). Its synthesis at a replication fork is required once on the leading strand and for the initiation of each Okazaki fragment on the lagging strand. The substitution of Polα by the Polδ holoenzyme is called "Pol switch" [Tsurimoto *et al.*, 1990; Waga and Stillman, 1994]. The Pol switch is mediated by the replication factor C (RF-C, RFC) and involves a complex interaction network of Polα, Polδ and RPA. RFC (also called clamp-loader)

binds preferentially to primer-template junctions in an ATP-dependent manner [Tsurimoto and Stillman, 1991; Yoder and Burgers, 1991], removes the Polα complex [Maga *et al.*, 2000] and promotes the loading of the sliding clamp - PCNA - onto the template DNA [discussed in: Waga and Stillman, 1998; Maga and Hübscher, 2003]. PCNA is a processive factor of both Polδ and Polε and is required for the complete assembly of Polδ holoenzyme and the stimulation of DNA synthesis [Lee and Hurwitz, 1990; Zhou *et al.*, 1997].

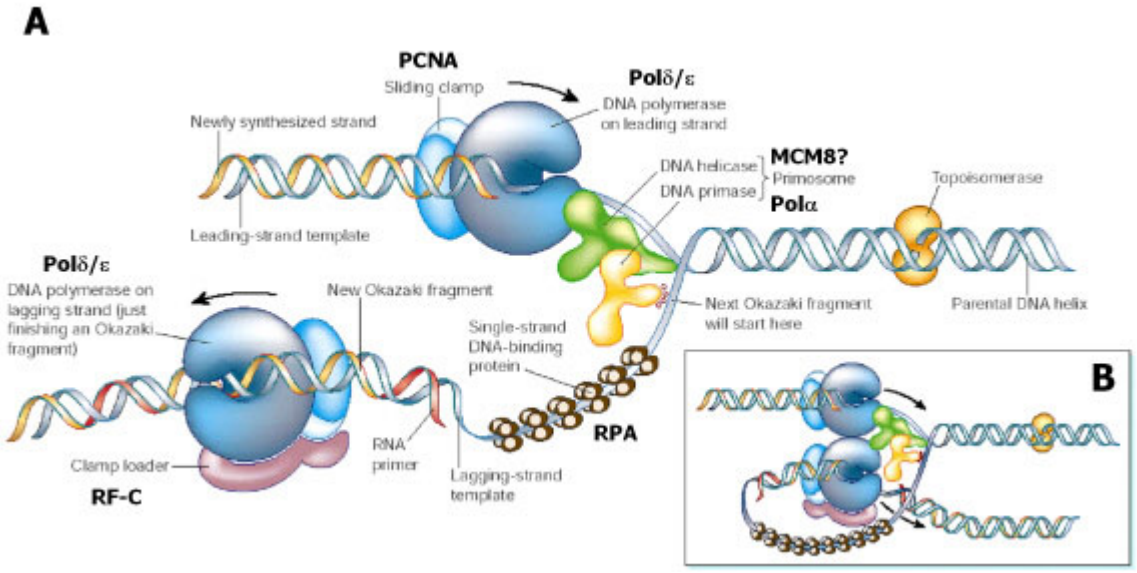


Figure 11: The eukaryotic replication fork
The replication fork is illustrated as a Y-shaped structure (**A**) but in reality, the fork is folded in three dimensions due to the homodimerisation of the replicative DNA polymerase holoenzyme (**B**). The parental DNA strands are unwound by a DNA helicase, powered by ATP hydrolysis. The DNA polymerase δ assisted by PCNA synthesises continuously the nascent DNA on the leading strand. The discontinuous DNA synthesis on the lagging strand is initiated by the Polα/primase producing an RNA/DNA primer which is extended by the replicative DNA polymerase δ until reaching the previous RNA/DNA primer (Okazaki fragment). Strand replacement at the 5' end of the previous Okazaki fragment leads to the formation of a flap structure which is further processed by Dna2 and Fen1 and finally ligated by the DNA ligase I. [The figure is adapted from: Alberts, 2003].

The Polδ as well as the Polε complex consist of one large catalytic subunit with an N-terminal proof-reading 3'-5' exonuclease domain and varying numbers of smaller ones with structural and regulatory functions [discussed in: Hübscher *et al.*, 2002]. These DNA polymerases are able to efficiently and accurately synthesise long stretches of DNA, shuttling thereby between their polymerisation and editing modes [reviewed in: Johnson, 1993]. Reaching the next Okazaki fragment, the polymerase holoenzyme displaces around 35 bp of the 5' end, which includes the RNA and DNA nucleotides polymerised by the error-prone Polα. In yeast, this flapped structure is bound by RFA

(RPA) and is subsequently removed by the nuclease RNase HI, Fen1 and Dna2 in a concerted action [Bae *et al.*, 2001; reviewed in: MacNeill, 2001]. Finally, the DNA gaps are joined by the DNA ligase I [Waga and Stillman, 1994].

Despite many years of replication studies, the function of Polδ and Polε in the synthesis of the leading or lagging strand is still heavily debated [discussed in: Kawasaki and Sugino, 2001]. In budding yeast the polymerase activity of Polε is not required for cell proliferation, denying a function in DNA synthesis [Kesti *et al.*, 1999]. On the other hand, the Polδ complex was proposed to act as a homodimer at the replication fork [Burgers and Gerik, 1998; Zuo *et al.*, 2000], thus permitting synthesis of both the leading and the lagging strand in a coordinated fashion (Figure 11). These findings suggest a major function of Polδ in DNA synthesis of both strands, whereas Polε may be required for replication initiation as well as for replication checkpoint signalling [Navas *et al.*, 1995].

1.6.4 Control and rescue mechanisms

The permanent surveillance of the integrity of the genome prior to and even more importantly during replication is crucial, in order to avoid any possibly deleterious DNA alteration [reviewed in: Toueille and Hübscher, 2004]. Molecular signalling cascades - so-called checkpoints [Hartwell and Weinert, 1989; discussed in: Melo and Toczyski, 2002] - respond to the presence of damaged or unreplicated DNA by triggering an arrest or a prolongation of the cell cycle mediated by CDKs [reviewed in: Nigg, 1995, respectively; Nyberg *et al.*, 2002]. In the G1-phase DNA damage checkpoints control the removal of potential mutagenic DNA lesions, preventing an entry of unrepaired damages into the S-phase. CDKs regulate the initiation of DNA replication (see above) and the assembly and activity of many of the replication fork proteins as well by modulating their phosphorylation state [discussed in: Frouin *et al.*, 2003; Henneke *et al.*, 2003]. During the S-phase there are at least three checkpoint cascades: 1) The replication checkpoint, which controls the origin firing and the integrity of the replication fork. 2) The S-M checkpoint that allows the exit from the S-phase upon completion of replication. 3) The intra-S-phase checkpoint, which signals DNA damages independently of replication [reviewed in: Myung and Kolodner, 2002; Bartek *et al.*, 2004]. Central roles in these S-phase checkpoint cascades are assigned to the human protein kinases ATM and ATR. ATM preferentially signals DSBs and other types of damage, whereas problems of replication fork progression are integrated by an ATR dependent cascade (see page 34) [discussed in: Abraham, 2001]. Several sensors are

proposed to signal replication problems to ATR: the replication proteins Polε, RPA, Dpb11 [discussed in: Bell and Dutta, 2002] as well as a RFC-like complex with RAD17 (in yeast Rad24) and the RAD9/RAD1/HUS1 complex (yeast Ddc1/Rad17/Mec3), which is structurally related to PCNA [Abraham, 2001].

S-phase CDKs tightly control replication initiation and prevent re-replication as well, thus continuous and accurate DNA synthesis, once started, is required at all replication forks [Nguyen *et al.*, 2001]. Any collapsed fork would inevitably lead to DNA loss and severe genomic rearrangements, when passing into cell division [Cox *et al.*, 2000]. Despite the rigorous control and removal of DNA damages prior to entry into the S-phase, some of them might have persisted or have arisen during replication due to external or internal genotoxic factors [Lindahl and Wood, 1999]. Stalling of the replisome can be caused by these DNA lesions, by its collision with RNA polymerases or by DNA synthesis problems. Cells have developed at least two mechanisms to avoid the fatal collapse of the replication fork: translesion synthesis and replication restart by HR [Hoeijmakers, 2001]. Relatively simple damages such as abasic sites or pyrimidine dimers can be bypassed by specialised translesion polymerases. These polymerases lack proof-reading activity and are therefore error-prone [reviewed in: Goodman, 2002; Prakash and Prakash, 2002; Rattray and Strathern, 2003]. Numerous studies in *E.coli* revealed multiple possible explanations how paused or stalled replication forks at more severe DNA lesions such as interstrand crosslinks or DSBs can be rescued [reviewed in: McGlynn and Lloyd, 2002; Michel *et al.*, 2004]. Some of them involve fork reversal, a process in which the nascent DNA strands pair and which depends on RecA, the bacterial orthologue of the HR protein Rad51 [McGlynn and Lloyd, 2001; McGlynn *et al.*, 2001; Singleton *et al.*, 2001]. Fork reversal leads to the formation of HJ, suggesting a functional relationship to repair by HR [Seigneur *et al.*, 1998]. In recent years, it has become evident that the same replication restart and damage bypass mechanisms exist in eukaryotic cells as well, although they may be of less importance than in bacteria. In checkpoint-deficient yeast mutants, the formation of HJ and the accumulation of ssDNA were observed at sites of stalled replication forks [Gruber *et al.*, 2000; Sogo *et al.*, 2002]. Indeed, Mec1 - the yeast counterpart of ATR - and Sgs1 play a central role in the stabilisation of stalled forks and in the promotion of replication restart [Lopes *et al.*, 2001; Tercero and Diffley, 2001; Cha and Kleckner, 2002; Cobb *et al.*, 2003; Bjergbaek *et al.*, 2005]. Failure of the checkpoint response resulted in DNA breaks [Myung *et al.*, 2001; Cha and Kleckner, 2002] and may account for the

observed chromosomal rearrangements in replication and recombination mutants [Chen and Kolodner, 1999].

Another characteristic of eukaryotic DNA replication is the spatial arrangement of several replisomes in a stationary replication factory, in which the replicating DNA is spooled through [discussed in: Lemon and Grossman, 2000; Frouin *et al.*, 2003]. Apart from accessory replication factors, a series of recombination proteins such as the yeast Rad52 group proteins [Lisby *et al.*, 2001; Essers *et al.*, 2002] or the human proteins WRN [Constantinou *et al.*, 2000] and BRCA2 [Lomonosov *et al.*, 2003] was found to co-localise with these replication factories and also to stabilise stalled replication forks. This suggests an intimate cooperation of the replication and recombination machinery during DNA synthesis. In addition, processing of stalled sites and replication restart depend on the presence of the *S.cerevisiae* Exo1 nuclease and on the *S.pombe* HJ resolvase complex Mus81-Eme1 [Doe *et al.*, 2002; Cotta-Ramusino *et al.*, 2005]. These observations strongly support the importance of homologous recombination-related mechanisms in keeping genome integrity during DNA replication [discussed in: Aguilera, 2001; McGlynn and Lloyd, 2002].

1.6.5 Plant cell cycle and replication

There are a lot of studies about the regulation of the plant cell cycle, which have revealed peculiarities such as endoreduplication that involves rounds of DNA replication without chromosome segregation and cytokinesis [reviewed in: De Veylder *et al.*, 2003; Dewitte and Murray, 2003]. In contrast, only sporadic and rather descriptive reports about plant DNA replication and its players are available. Replication origins of plants are not mapped but there are a few reports about plant ORC proteins from corn [Witmer *et al.*, 2003] and the Arabidopsis ORC2 which turned out to be essential for embryo development [Collinge *et al.*, 2004], indicating a functionally conserved replication initiation. The cloning and the expression of the CDC6 homologues of Arabidopsis [Castellano *et al.*, 2001; Ramos *et al.*, 2001] and of tobacco [Dambrauskas *et al.*, 2003] were reported. Members of the MCM complex were described for tobacco, corn and Arabidopsis [Springer *et al.*, 1995; Sabelli *et al.*, 1996; Dambrauskas *et al.*, 2003]. The best studied plant MCM-encoding gene is *PROLIFERA*, the Arabidopsis homologue of yeast Mcm7. It was found in a screen for essential genes of gametophyte and embryo development [Springer *et al.*, 1995; Springer *et al.*, 2000]. CDC45 was shown to participate in the assembly of replication forks. Arabidopsis *CDC45*-RNAi alleles were generated and revealed a meiotic but no somatic phenotype.

The observed SPO11-independent fragmentation of meiotic chromosomes may originate from incomplete initiation of pre-meiotic replication [Stevens *et al.*, 2004]. Although a thorough analysis of their biological importance remains to be done, the discovery of plant homologues of replication initiation factors underline the existence of a conserved mechanism.

Classically, DNA polymerase α and δ activities were isolated and classified according to their biochemical properties from corn [Coello and Vazquez-Ramos, 1995; Gomez Roig and Vazquez-Ramos, 2003] and from the wheat germ systems [Richard *et al.*, 1991; Laquel *et al.*, 1993; Benedetto *et al.*, 1996]. Recently, the rice genes coding for the homologues of the catalytic subunit of Polα, of two subunits of Polδ and of the replication accessory proteins FEN1 and PCNA were cloned and analysed for their spatial expression [Yokoi *et al.*, 1997; Kimura *et al.*, 2001; Uchiyama *et al.*, 2002]. These reports propose highly conserved functional and enzymatic similarities between the DNA replication of plants and other species. Similarly, the replication rescue mechanisms may exist also in plant as indicated by the discovery of an Arabidopsis homologue of the human translesion polymerase Polκ [Garcia-Ortiz *et al.*, 2004]. Moreover, Arabidopsis homologues of the S-phase checkpoint kinases ATR and ATM were described. Plants mutated in *ATR* revealed hypersensitivity to various genotoxic treatments and failure of cell cycle arrest, triggering apoptotic phenotypes [Culligan *et al.*, 2004]. The DSB signalling kinase *ATM* was found to be expressed ubiquitously and independently of DNA damage and to influence the expression levels of several repair-related genes [Garcia *et al.*, 2000; Garcia *et al.*, 2003]. In agreement with its proposed biological function to promote a physiological response to DSBs, mutations in *ATM* yielded increased sensitivity to γ-radiation and to exposure to MMS and were also impaired in meiosis (see page 48) [discussed in: Eckardt, 2003; Garcia *et al.*, 2003].

1.7 Meiotic Recombination

Meiosis is a key event in the life cycle of all sexually reproducing eukaryotic organisms, initiating the transition from the diploid to the haploid generation [discussed in: Zickler and Kleckner, 1999; Petronczki *et al.*, 2003]. The reductional meiotic division consists of the separation of the parental chromosomes in meiosis I (4N→2N) and of the sister chromatids in meiosis II (2N→1N). Whereas meiosis II resembles a mitotic division, meiosis I is characterized by specific chromosomal figures and nuclear structures. They were thought to be quite conserved within all kingdoms but many species-specific variations were recently discovered [reviewed in: Shaw and Moore, 1998; Zickler and

Kleckner, 1999; Villeneuve and Hillers, 2001; Petronczki *et al.*, 2003]. In most species the alignment of the maternal and paternal chromosomes and recombination between homologous sequences are mechanistically linked, resulting in full synapsis mediated by the proteinaceous synaptonemal complex (SC). This means that mutations in genes with early functions in recombination abolish the formation of the SC, provoking meiotic defects and/or arrest. Analysis of one of the best studied meiotic systems - budding yeast - revealed that the somatic DNA repair by HR and the meiotic recombination process share most of the proteins (Figure 12); *S.cerevisiae* strains depleted for proteins, mechanistically involved in processing DSBs and in repair via the HR pathway, frequently also show severe meiotic phenotypes [Roeder, 1997; Sung *et al.*, 2000].

1.7.1 Enzymology of meiotic recombination

The induction of DSBs by the yeast endonuclease Spo11 initiates meiotic recombination at a high frequency [Keeney *et al.*, 1997; Keeney, 2001]. Orthologues of the Spo11 are found in most organisms, presumably carrying out the universal function of introducing DNA DSBs to meiotic chromosomes [discussed in: Villeneuve and Hillers, 2001]. Spo11 shows some homology to the A subunit of the archaebacterial topoisomerase VI complex which induces temporary breaks in order to disentangle DNA. In budding yeast meiosis, the Mre11/Rad50/Xrs2 complex is assumed to participate in the Spo11-dependent induction of DSBs and also in the processing of the ends.

In yeast, the recombination proteins Rad51 [Shinohara *et al.*, 1992] and its meiotic paralogue Dmc1 [Bishop *et al.*, 1992] assemble to a nucleoprotein complex with the single-stranded ends [Shinohara *et al.*, 2000]. The loading of the nucleoprotein filaments is facilitated by the proteins of the yeast Rad52 epistasis group [Sugiyama and Kowalczykowski, 2002; Symington, 2002; Sung *et al.*, 2003] or by the mammalian RAD51 paralogues [Masson *et al.*, 2001]. Key players in meiotic recombination of yeast are also the chromatin remodelling ATPases Rad54 and its meiotic paralogue Rdh54. They have distinct functions in strand invasion between homologous chromosomes, nucleoprotein filament disassembly and branch migration [Shinohara *et al.*, 1997; Shinohara *et al.*, 2000; Alexiadis and Kadonaga, 2002; Solinger *et al.*, 2002; Alexeev *et al.*, 2003; Tan *et al.*, 2003]. Some eukaryotic homologues of the bacterial mismatch repair proteins MutS [Pochart *et al.*, 1997; Edelmann *et al.*, 1999; Zalevsky *et al.*, 1999] and MutL [Baker *et al.*, 1995; Baker *et al.*, 1996; Edelmann *et al.*, 1996; Hunter and Borts, 1997] are anticipated to promote

crossover, branch migration and the resolution of the Holliday junctions [Roeder, 1997; Kolodner and Marsischky, 1999].

In fission yeast, the HJs of meiotic chromosomes may be mainly resolved by the Mus81/Eme1 nuclease complex [discussed in: Hollingsworth and Brill, 2004], whereas distinct resolvase activities participate in this process in other organisms. The budding yeast Sgs1/Top3 complex [Fabre *et al.*, 2002] and its human counterpart BLM/TOPOIIIα [Wu and Hickson, 2003] were also reported to contribute in later steps of HR such as HJ processing. Finally, a role in HJ resolving was assigned to the RAD51 paralogues RAD51C and XRCC3 [Constantinou *et al.*, 2001; Liu *et al.*, 2004].

1.7.2 Meiotic recombination in Arabidopsis

Plant genes and the effect of their mutations on various aspects of meiosis have been described extensively [Bhatt *et al.*, 2001; Caryl *et al.*, 2003]. Meiotic HR is relatively well studied in Arabidopsis but the biological roles of plant orthologues of some key players such as the Rad54-like ATPase or the Rad51 paralogues remain to be elucidated (Figure 12). Reverse genetic approaches analysing the Arabidopsis homologues of known yeast or mammalian HR-related genes mostly yielded meiotic phenotypes. In some cases a function of these proteins in DNA repair was indicated by an enhanced sensitivity to genotoxic treatments but their roles in somatic HR remains to be shown. These findings indicate that HR is a minor DSB repair pathway in somatic plant cells but essential for meiosis (Chapter 1.8.1, page 54).

In contrast to other species the Arabidopsis genome codes for three paralogues of Spo11 and also a homologue of the archaebacterial B subunit of topoisomerase VI (TOP6B) [Hartung and Puchta, 2000; Hartung and Puchta, 2001]. A mutation of the *SPO11-1* gene severely reduced the formation of chiasmata and bivalents, abolishing full synapsis [Hartung and Puchta, 2000; Grelon *et al.*, 2001]. Meiotic division proceeded in the *spo11-1* plants, resulting in “polyads” of random DNA content instead of typical tetrads. It remains to be elucidated whether the residual DSBs and formation of few chiasmata is due to alternative pathways and/or to the function of the two other SPO11 paralogues. However, both of them but not *SPO11-1* were shown to interact with TOP6B, suggesting a eukaryotic topoisomerase-like function (also see page 3) [Hartung and Puchta, 2001].

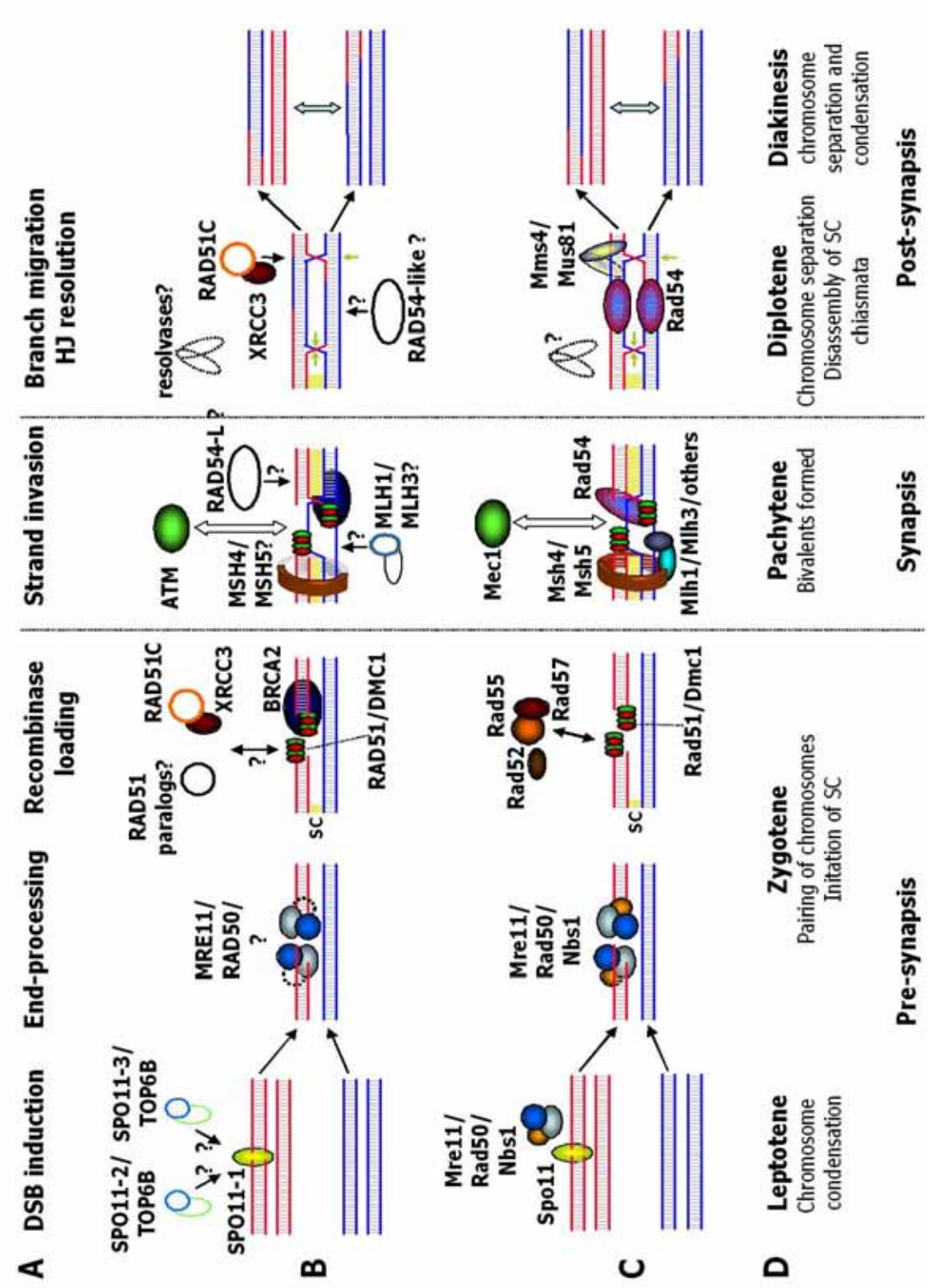


Figure 12: The mechanism of meiotic recombination
The key steps of meiotic recombination (**A**) are mediated by a series of Arabidopsis (**B**) and yeast (**C**) proteins, which were also shown to be involved in mitotic repair by homologous recombination (Chapter 1.4.1). The temporal processing of the Spo11-induced DSB is crosstalking with the chromosome choreography of meiosis (**D**). The involvement of proteins in meiotic HR is reported (colored and filled shapes), suggested (empty shapes) or unknown (dashed shapes). [Adapted from: Schuermann *et al.*, 2005].

Mutants of the Arabidopsis orthologues of MRE11 [Puizina *et al.*, 2004] and RAD50 [Gallego *et al.*, 2001; Bleuyard *et al.*, 2004b] were isolated and analysed for their meiotic phenotypes. Both mutants exhibited chromosomal fragmentation and failure of synapsis, supporting a role in early steps of meiotic homologous recombination for the Arabidopsis MRE11/RAD50 complex, presumably in processing of DNA ends [Alani *et al.*, 1989; Ohta *et al.*, 1998]. In contrast to the yeast model, the protein complex is not required for induction of the SPO11-mediated DSBs, since the meiotic chromosome fragmentation and the complete sterility of *mre11* could be partially suppressed by *spo11-1* mutations.

Consistent with the yeast model, Arabidopsis mutants of *DMC1* [Couteau *et al.*, 1999] and *RAD51* [Li *et al.*, 2004b] were severely affected in meiotic recombination. Their chromosomes clearly failed to synapse and to form bivalents but unlike in yeast only *rad51* mutants exhibited chromosome fragmentation, most likely provoked by persisting meiotic DSBs. This suggests a function of DMC1 in the selective invasion of the homologous chromosome rather than in the repair of the SPO11-induced DSBs [Masson *et al.*, 2001]. In the *dmc1* background remaining breaks could be repaired by RAD51-mediated HR between sister-chromatids and the lack of joint molecule formation prevents synapsis. Recently, the function of these two recombinases was suggested to be dependent on their genetic and molecular interaction with the two Arabidopsis paralogues of BRCA2. The absence of synapsis and the chromosome fragmentation phenotype of RNAi-*BRCA2* allele was reminiscent of that of the double-mutant *dmc1*/RNAi-*RAD51* [Siaud *et al.*, 2004], and both BRCA2 homologues interacted with DMC1 and RAD51 in a yeast-two-hybrid analysis. With the same technique interactions within the RAD51 paralogues were found: between RAD51 and XRCC3 as well as between XRCC3 and RAD51C [Osakabe *et al.*, 2002].

Indeed, *xrcc3* mutant plants revealed SPO11-1-dependent meiotic defects and sterility [Bleuyard *et al.*, 2004a; Bleuyard and White, 2004]. In contrast to the previously described mutants, synapsis appeared to be unaffected and bivalents were formed normally. As meiosis proceeded chromosome bridges and fragmentation were observed suggesting a distinct role of XRCC3 in a later phase of meiotic recombination, most likely in branch migration or in HJ resolution in collaboration with RAD51C as proposed in mammalian meiosis [Liu *et al.*, 2004]. Arabidopsis encodes a series of homologues of bacterial mismatch repair proteins MutS [Ade *et al.*, 1999; Culligan and Hays, 2000] or MutL [Jean *et al.*, 1999]. A mutation in the Arabidopsis *MSH4* gene provoked a reduction of chiasmata number and also a delayed and incomplete

synapsis, which resulted in the appearance of univalents [Higgins *et al.*, 2004]. This supports an early role of MSH4 in meiotic recombination, probably in a heterodimeric complex with MSH5. There it could form a sliding clamp which keeps together the Holliday junctions and the homologous chromosomes, as proposed recently for the human MSH4/MSH5 complex [Snowden *et al.*, 2004].

An interesting aspect of plant meiosis is the lack of a checkpoint cascade which controls the progression of meiosis, provoking meiotic arrest in yeast and apoptosis in animals. All the mutants described above proceed through meiosis despite containing aberrant chromosome figures. Nevertheless, the Arabidopsis homologue of mammalian ATM was shown to be involved in somatic DNA damage response (Chapter 1.5.1, page 33) [Kastan and Lim, 2000] and in meiotic recombination as well; mutants in this gene revealed a chromosome fragmentation phenotype similar to that of *xrcc3* mutant [Garcia *et al.*, 2003]. This suggests that Arabidopsis ATM solely controls the meiotic DSB repair by HR but not the integrity of meiotic chromosomes. MEI1 is another protein that was anticipated to have a meiotic checkpoint function. Mutations of *MEI1* caused severe meiotic defects but no effects in somatic tissue [Grelon *et al.*, 2003]. The MEI1 protein seems to act upstream of SPO11-1, since its chromosome fragmentation phenotype is independent of induction of DSBs, suggesting a function in the control or repair of the pre-meiotic DNA synthesis.

1.8 Rationale for this work

Compared to yeast and mammalian systems little is known about DNA replication and repair mechanisms in plants. Although a considerable amount of data using forward and reverse genetic approaches has accumulated in recent years, many questions remain to be answered. There are speculations about the reasons for the inefficient use of HR for DSB repair and many more studies in plants are needed to elucidate the DNA metabolism of plants (see below). The phase of cell cycle, the chromatin structures and also the availability of the enzymatic machinery are anticipated to influence DNA repair by HR. The development of reliable transgenic substrates to monitor repair events by HR facilitated a series of studies on environmental impacts on the plant genome. The same tools can also be used to study endogenous factors influencing the efficiency of HR. This enables a genetic screen for new plant genes which are able to modulate the frequency of somatic HR (see below). Novel genes identified by such a screening approach may lead to the better understanding of plant

genome maintenance mechanisms which can result in the improvement of applied aspects of plant science such as gene targeting (see below).

In our laboratory, it was intended to study Arabidopsis genes which influence and modulate genome stability and DNA repair. Forward genetic screens were performed in order to determine plant factors that alter the somatic frequency of HR [Molinier *et al.*, in prep]. Dominant alleles were created by activation-tagging and their effect on HR could be directly assessed using transgenic HR substrates (see below). By this approach the Arabidopsis mutant line *hw17* was isolated, exhibiting a moderate increase of somatic HR. The aim of this study was the throughout characterisation of this mutant lines. The impact of the mutated gene on the genome stability of Arabidopsis was analysed and highlighted an intimate connection of homologous recombination, DNA replication and the maintenance of genome integrity.

1.8.1 Repair DNA double-strand breaks: HR versus NHEJ

Classically, it was assumed that DSBs are predominantly repaired by HR in lower eukaryotes and by NHEJ in higher eukaryotes [Sargent *et al.*, 1997]. The accuracy of these two DSB repair pathways significantly contributes to the genome stability of the organism. However, recent findings question this paradigm. Depletion of mouse *RAD51* and *RAD54* resulted in embryo lethality and hypersensitivity to DSB induction, respectively, implying a crucial role of HR in the development of multicellular organisms or in DNA repair [Tsuzuki *et al.*, 1996; Essers *et al.*, 1997]. In a direct comparison of pathway utilisation upon DSB induction in cultured mammalian cells, 30-50% of the repair events were assigned to HR, the remaining ones were repaired by NHEJ, often accompanied by small insertions or deletions [Liang *et al.*, 1998]. Unfortunately, no comparable assessments of pathway utilisation are available for plants but the viability and normal phenotype of Arabidopsis *rad51* mutants suggests substantial differences to the mammalian system [Li *et al.*, 2004b].

In a genome region of budding yeast in which DSB repair by both pathways could be monitored, the recruitment of NHEJ proteins was found to precede that of HR factors and presumably to result in the stabilisation of broken DNA ends [Frank-Vaillant and Marcand, 2002]. Commitment to HR is then marked by end processing and the recruitment of HR factors [Aylon *et al.*, 2003]. Proteins of the structural maintenance of chromosome (SMC) family are involved in many aspects of DNA metabolism such as cohesion of sister-chromatids after replication and repair [reviewed in: Jessberger, 2002; Hagstrom and Meyer, 2003; Lehmann, 2005]. Yeast SMC1 was proposed to

promote the commitment to HR by engaging Rad52 or Rad54 [Schär *et al.*, 2004]. The G2-phase is the most prominent in the life cycle of yeast, in which the post-replicative sister-chromatids are typically aligned, mediated by complexes with SMC proteins. The cohesion of sister-chromatids in G2 ensures a proper segregation of chromosomes in mitosis but the proximity of template sequences also facilitates repair by HR. In contrast, terminal mammalian as well as somatic plant cells arrest in the pre-replicative G1-phase (termed G0). This means that templates for repair by HR are only available on the presumably distant homologous chromosome or as repeated sequences at ectopic positions. The use of the latter necessarily leads to substantial sequence loss, which may be even more deleterious than the introduction of small genome alterations by NHEJ. In conclusion, the choice for either repair pathway is largely depending on the cell cycle phase: During the S- and G2-phase most DSBs are repaired by HR, whereas they are rejoined by NHEJ in the G1-phase [discussed in: Khanna and Jackson, 2001].

Another level of pathway decision may be the availability of repair factors. In meiotic cells, transcriptional control of DSB repair genes influences the decision of cells to use HR or NHEJ; components of NHEJ are down-regulated in mammals as well as in budding yeast [Goedecke *et al.*, 1999; Haber, 2000]. In support of this hypothesis, ectopic expression of the bacterial HR proteins RecA in Arabidopsis [Reiss *et al.*, 1996; Reiss *et al.*, 2000] and of RuvC in tobacco [Shalev *et al.*, 1999] was shown to stimulate the somatic HR frequencies. Similarly, over-expression of human RAD52 in cultured monkey cells yielded an increased HR frequency and radiation resistance [Park, 1995]. In contrast, depletion of RAD54 in mice or chicken cell lines reduced HR levels for DSB repair, the gene targeting frequency and the resistance to ionising radiation [Bezzubova *et al.*, 1997; Essers *et al.*, 1997].

Yeast cells accurately and efficiently repair DSBs in their genome by HR, preferentially according to the DSBR model. The knowledge of repair by other pathways, especially by NHEJ, mostly originates from HR-deficient cells [discussed in: Paques and Haber, 1999]. Accordingly, HR-mediated DSB repair in human cells was found to be stimulated by the depletion of the NHEJ factor DNA-PK [Allen *et al.*, 2002]. Based on these observations it was proposed that a diminution of one of the DSB repair pathways favours the use of the other [discussed in: Ray and Langer, 2002]. In plants, there is one reported attempt to stimulate HR by knocking-out the NHEJ factor KU80 [Gallego *et al.*, 2003]. Although *ku80* plants exhibited enhanced sensitivity to a variety of genotoxic treatments [West *et al.*, 2002; Friesner and Britt, 2003; Gallego *et*

al., 2003] suggesting impaired repair, no increase of intra-molecular HR was observed. Therefore, it is hypothesised that an alternative repair pathway for DSBs may exist in plants.

In somatic tobacco and maize cells the frequencies of DSB repair by ectopic or allelic HR was estimated at one in about 10,000 events [Shalev and Levy, 1997; Puchta, 1999; Gisler *et al.*, 2002]. The measurement of inter-molecular DNA exchanges at a given transgenic locus yielded three to four times more HR events in homo- than in hemizygous plants. This indicates the frequent use of the homologous chromosomes and not of the sister-chromatid which is available only in the G2-phase and thus of low abundance in plants [Molinier *et al.*, 2004b]. Repeated sequences in close proximity to the DSB can be engaged to repair by accurate DSBR or by mutagenic SSA (Chapter 1.4.1.2, page14). Molecular analysis of HR events in this sequence context revealed a two orders of magnitude more frequent use of SSA accompanied by the deletion of the intervenient sequences [discussed in: Puchta, 2005].

However, the vast majority of somatic DSBs are repaired by the NHEJ or a related pathway. These repair events are mostly accompanied by a broad variety of extensive sequence deletions and/or insertions [Gorbunova and Levy, 1997; Salomon and Puchta, 1998; Kirik *et al.*, 2000; Lloyd *et al.*, 2005], which were also reported for mammalian cells [Pipiras *et al.*, 1998]. This is in sharp contrast to NHEJ in budding yeast, in which most breaks are repaired in an errorless fashion and only rarely short mitochondrial or retrotransposon sequences are found to be inserted at fixed break sites [Moore and Haber, 1996; Teng *et al.*, 1996; Ricchetti *et al.*, 1999]. The insertion of ectopic filler DNA by DSB repair of plants can be explained by a mechanism related to SDSA (termed one-sided invasion, OSI) [Puchta, 1998]. The resected ssDNA invades duplex DNA at a random genomic position with micro-homology. The short base pairing is sufficient to prime DNA synthesis and its elongation copies the ectopic sequence. After release from the template the lack of homology with the second strand favours the joining of the DNA end by a NHEJ-related mechanism. In fact, patches of micro-homologies are often found at the second junction of the ectopic sequence, at repaired sites with sequence deletions or in T-DNA integration events [Rinehart *et al.*, 1997; Salomon and Puchta, 1998; Tzfira and Citovsky, 2003]. Due to its dependence on small stretches of homology to seal the break, this type of repair was termed SSA-like and may represent an intermediate mechanism between NHEJ and HR, of which the molecular players are largely unknown [discussed in: Gorbunova and Levy, 1999]. However, the junctions of vertebrate NHEJ repair events resemble the plant ones,

indicating a conserved fashion of dealing with strand breaks in higher eukaryotes [Lehman *et al.*, 1994; Nicolas *et al.*, 1995; Mason *et al.*, 1996; Pelczar *et al.*, 2003].

The predominant fashion of somatic DSB repair by error-prone homology-dependent or -independent mechanisms significantly influences the integrity and evolution of the plant genome particularly in respect to the late determination of the germline (see page 3). Apart form transposable elements [Bennetzen, 2000], the way and the accuracy of DSB repair contributes significantly to genome size. Indeed, significantly longer insertions and smaller deletions were reported for tobacco compared to Arabidopsis [Kirik *et al.*, 2000; Orel and Puchta, 2003], the genome size of which is about 20 times smaller [Bennett and Leitch, 1997]. This intrinsic difference in DNA metabolism between different species may be a general driving force for the evolution of genome size and thus of organisms [discussed in: Petrov, 2001].

1.8.2 Targeted modifications of the plant genome

The introduction of foreign genes depends on the DSB repair machinery of the host. Gene targeting (GT) defines the replacement or the change of a host gene by an ectopic template. This process depends on repair by HR through the formation of a double crossover between the target gene and the flanking homologous sequences provided on the template [reviewed in: Reiss, 2003]. Quite some time ago, the feasibility of GT in plants was demonstrated in tobacco cells, using different experimental setups [Paszkowski *et al.*, 1988; Lee *et al.*, 1990; Offringa *et al.*, 1990]. However, the prevalence of NHEJ in somatic plant cells mostly results in transformation events with random and complex integration sites, even when extensive homologous sequences were provided (see above). Recent estimations in Arabidopsis [Kempin *et al.*, 1997; Hanin *et al.*, 2001] and rice [Terada *et al.*, 2002] suggested a ratio of GT to random integration in the range of 10^{-4} to 10^{-6}. This frequency is far too low for an efficient use of GT for both applied and academic purposes. Presumably, the stimulation of HR repair in cells destined for the targeting event could positively influence the GT frequency. Therefore, a lot of effort was put into the development of efficient targeting systems and into the discovery of factors that modulate HR frequency [discussed in: Britt and May, 2003; Hanin and Paszkowski, 2003]. Disappointingly, a first attempt to increase GT efficiency by ectopic expression of the bacterial Rad51 homologue RecA failed, despite the observed stimulation of somatic HR [Reiss *et al.*, 2000]. But many more Arabidopsis mutants with increased HR level remain to be tested for their influence in GT [discussed in: Schuermann *et al.*, 2005].

1.8.3 Assessing HR frequency in plants

In Arabidopsis, many genetic markers both of phenotypic or molecular nature are known and mapped (see http://arabidopsis.org). They can be used to estimate meiotic recombination frequencies between given loci. PCR-based techniques even allow the rapid genome-wide mapping of recombination events [Peters *et al.*, 2001]. They always reflect the meiotic division of the previous generation, in which it would have been difficult to molecularly characterise an event. However, somatic HR events are rare and occur randomly in a single determined cell unlikely to be clonally amplified or transmitted to the following generation, making its molecular analysis a hard task. Classically, these problems were circumvented by the use of artificial recombination substrates, which were based in selectable marker genes. Recombination events conferred resistant calli derived from tobacco protoplasts and could be analysed [Baur *et al.*, 1990; Offringa *et al.*, 1990; Peterhans *et al.*, 1990; de Groot *et al.*, 1992; Tovar and Lichtenstein, 1992; Hrouda and Paszkowski, 1994]. Artificial recombination substrates consist of a reporter gene, which is split into two non-functional parts with overlapping homologous sequences. They are either provided extra-chromosomally on plasmid DNA or as integrated transgene [reviewed in: Reiss, 2003]. In contrast to the prerequisite of about 50 nt homology in yeast, efficient extra-chromosomal HR in plants required more extended complementary sequences and was found to preferentially happen according to the SDSA model [Puchta and Hohn, 1991a; Puchta and Hohn, 1991b].

Later on, transgenic Arabidopsis and tobacco substrate lines were developed, in which a HR event restores a functional *β-Glucuronidase* (*GUS*, *UidA*) [Swoboda *et al.*, 1994; Puchta *et al.*, 1995a] or firefly *Luciferse* (*LUC*) gene [Kovalchuk *et al.*, 2003; Fritsch *et al.*, 2004]. These systems possess several advantages over selectable marker gene substrates, since tissue culture artefacts can be avoided and the HR events can be easily visualised and assessed in the whole plant by an *in situ* enzymatic assay, in the latter case even non-destructively. In addition to the artificial HR substrates, a convenient natural system exists in a tobacco ecotype, which contains a semi-dominant mutation in the *sulfur* (*su*) locus controlling the chloroplast pigmentation [Gorbunova *et al.*, 2000]. Hemizygous plants are pale green and show occasionally so-called twin spots; adjoining dark green (*Su*/*Su*) and white sectors (*su*/*su*), which are believed to derive from recombination between homologous chromosomes.

Depending on the linear arrangement of the two non-functional parts, the restoration of a functional marker gene reflects recombination events within the same

chromosome (intra-molecular) or between sister chromatids and homologous chromosomes (inter-molecular) (see Figure 13). Arabidopsis lines with different arrangements of the HR marker were used in several studies: 1) Direct repeats of the homologous sequences separated by a spacer visualise intra- and inter-molecular as well as SSA recombination events [Gherbi *et al.*, 2001]. 2) Direct repeats oppositely orientated detect primarily inter-molecular recombination, since intra-molecular HR produces an instable circular DNA [Molinier *et al.*, 2004b]. 3) Restoration of the marker in substrates with indirect repeats solely reflects intra-molecular recombination [Gherbi *et al.*, 2001; Lucht *et al.*, 2002]. Based on these substrate lines the frequency of HR events in a given locus is estimated to be about 10^{-6} per cell, although some variation was reported, presumably depending on the chromosomal position of the HR substrate [discussed in: Puchta and Hohn, 1996].

1.8.4 A genetic screen for altered HR frequency

Genetic screens for altered phenotypes are powerful tools to identify novel genes [discussed in: Page and Grossniklaus, 2002]. In budding and fission yeast, the screening for altered ionising and UV radiation sensitivity (*Rad* genes) contributed enormously to the current knowledge of eukaryotic DNA repair. In the model plant Arabidopsis, similar attempts were undertaken in order to isolate genes conferring tolerance to X-rays [Masson *et al.*, 1997], to UV irradiation [Jenkins *et al.*, 1995], to γ-rays [Davies *et al.*, 1994] or to the radio-mimicking agent methyl methanesulfonate (MMS) [Revenkova *et al.*, 1999].

Due to their sensitivity to DSB induction, some of the mutants from this X-ray screen (*xrs* mutants) - not mapped yet - were analysed for their impact on somatic and meiotic recombination. They revealed interesting divergent behaviour; *xrs4* plants revealed reduced somatic but increased meiotic HR frequencies, whereas in *xrs9* both are decreased and in *xrs11* no HR response was observed upon DSB induction [Masson and Paszkowski, 1997]. The screens for UV and γ-ray sensitivity (*uvr*, *uvh*) resulted in the isolation of some plant homologues of NER and photoreactivation proteins (see pages 28 and 31). Finally, the screen for MMS hypersensitivity yielded two mutants with altered somatic HR frequencies. A decrease was reported for *mim* mutants, whereas the over-expression of this SMC-like gene increased the level of somatic HR, suggesting a regulatory function of MIM in DSB repair [Mengiste *et al.*, 1999; Hanin *et al.*, 2000].

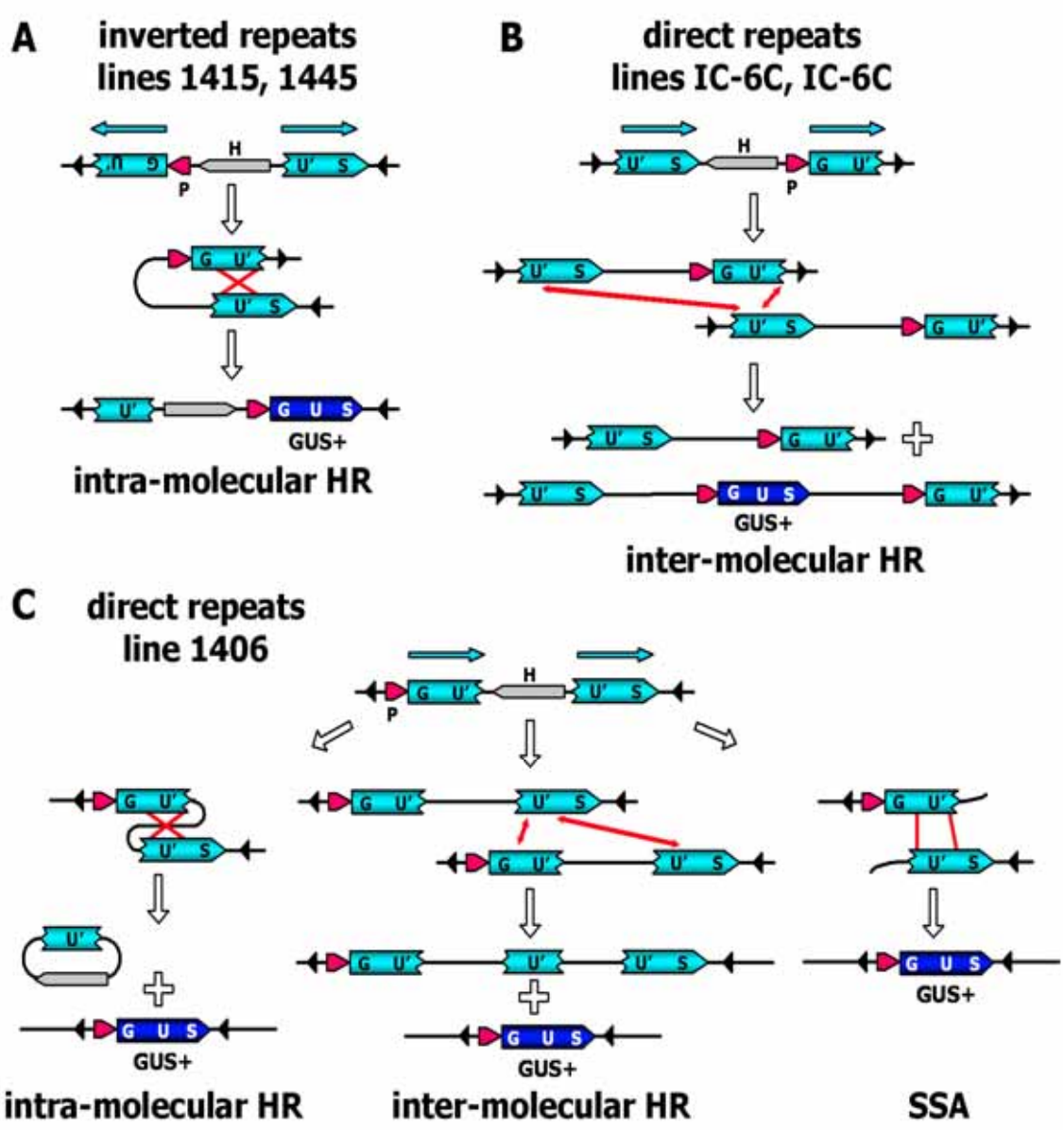


Figure 13: Artificial homologous recombination substrates in Arabidopsis lines

The integrated artificial recombination substrates with 618 bp homologous sequences (U') monitor HR events based on the functional restoration of the *β-Glucuronidase* gene (GUS+). The spatial arrangement of the repeats defines the molecular processes which lead to a detectable homologous recombination event. **A.** The substrate with indirect repeats results in intra-molecular HR without any loss of sequences. **B.** The substrate with direct repeats detects inter-molecular HR events by gene conversion or unequal reciprocal recombination. Intra-molecular recombination would form an extra-chromosomal circular DNA with GUS+, which presumably is quickly degraded but its re-integration into the genome has been also observed [Molinier *et al.*, 2004b]. **C.** Recombination events between the direct repeats of the substrate following different molecular mechanisms can result in the restoration of functional GUS+: intra- as well as inter-molecular recombination in a RAD51/ (Rad52)-dependent manner (left and middle panel) and the SSA type, mediated by RAD1 (right panel). The inter-molecular HR leads to gene conversion or reciprocal exchange, whereas the intra-molecular events necessarily result in the loss of the intervenient sequences. Black triangles: T-DNA borders; P: CaMV promoter; H: hygromycin resistance gene. [This figure is adapted from: Schuermann *et al.*, 2005].

Interestingly enough, yeast Smc1 was recently shown to influence the pathway choice between HR and NHEJ at DSBs [Schär *et al.*, 2004]. Similarly, yeast Smc6 was anticipated to be involved in inter-chromosomal and sister-chromatid recombination [Onoda *et al.*, 2004]. The analysis of the second MMS-sensitive mutant *bru* revealed increased somatic HR, release of transcriptional gene silencing as well as a pleiotropic growth phenotype. Therefore, a cooperative role of BRU in replication and stabilisation of the chromatin structure was proposed [Takeda *et al.*, 2004].

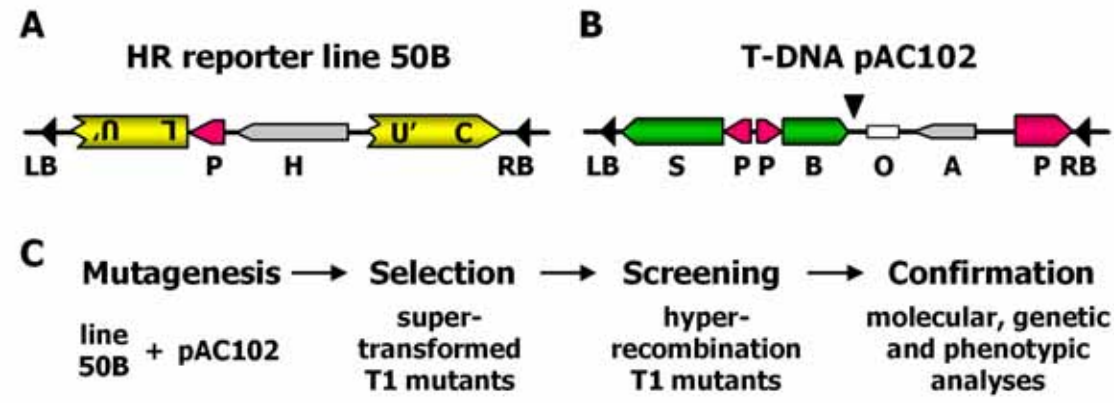


Figure 14: The design of the genetic screen for increased HR frequency
A. Scheme of the intra-molecular homologous recombination substrate; introduced into Arabidopsis by *A.tumefaciens*-mediated transgenesis yielding the HR reporter line 50B. **B.** Scheme of the T-DNA that was used to mutagenise the HR reporter line 50B, containing the strong viral CaMV 35S promoter for activation-tagging of genes near the integration site. **C.** Screening procedure to find Arabidopsis mutants with increased intra-molecular HR frequencies. A, bacterial *ampicilin resistance* gene; B, *bar* gene, confers resistance to phosphinotricin; H, *hygromycin resistance* gene; LB, T-DNA left border sequence; O, bacterial origin of replication; P, CaMV 35S promoter sequence; RB, T-DNA right border sequence; S, *tp-sul* gene, confers resistance to the herbicide sulfonamide; the arrow-head indicates the *Hin*dIII restriction site which can be used to isolate the pAC102 T-DNA integration site by plasmid rescue.

The screens for increased sensitivity to DNA-damaging agents resulted indirectly in the description of some plant mutants with altered HR frequencies. The use of Arabidopsis lines with transgenic HR substrates (Figure 13) allows the monitoring of HR events of individual plants and thereby a direct screening for altered frequency. With these tools in hand, a forward genetic screen was initiated. Due to the non-destructive assessment of HR events in firefly *Luciferase*-based HR substrates, a single copy reporter line (50B) was selected as genetic background for mutagenesis. This substrate monitors homologous recombination events between the two inverted repeats of the *luciferase* gene on the same chromosome (intra-molecular) (Figure 14A). An average of about two recombination events per plant was observed in this line, when assessing at the stage of fully developed rosettes [Fritsch, 2004]. This substrate line was mutagenised

by random insertion of Agrobacterium T-DNAs, which contained an activation-tag proximal to the T-DNA right border (RB) sequence (Figure 14B and Plasmid 1, page XIV) [extensively described in: Fritsch, 2004].

The aim of this thesis was the molecular characterisation of one of the mutant plants, isolated in the genetic screen for increased somatic intra-molecular HR. The mutated gene was identified and its cDNA cloned. The causality of the mutation with the HR phenotype was proven by genetic and molecular means. Based on the scientific background discussed above, the isolated gene should be placed into the context of described processes of DNA metabolism. Its tentative contribution to the regulation of HR is analysed and discussed.

Chapter 2 Results

2.1 Characterisation of the recombination mutant *hw17*

2.1.1 Analysis of the T1 generation

In a genetic screen for dominant mutations in Arabidopsis that affect the frequencies of somatic homologous recombination, the mutant line *hw17* was isolated. This T1 plant (Figure 15A) was classified into a mutant group exhibiting a 15 to 50 fold increase in the frequency of somatic homologous recombination [Fritsch, 2004].

A callus culture derived from two leaves of the *hw17* mutant served as backup material in case of sterility and to isolate sufficient DNA for the molecular analysis of the T1 generation. Genomic DNA was digested with *Hin*dIII, blotted and hybridised with a probe for the *Ampicilin* gene of the mutagenising T-DNA. Two integration sites were detected by Southern blot analysis (Figure 15B). The estimated molecular weights of these bands were about 5kb and 13kb, respectively. For further distinction, the two integration sites were named *hw17A* and *hw17B* for the locus corresponding to the 13kb and to the 5kb *Hin*dIII fragment, respectively.

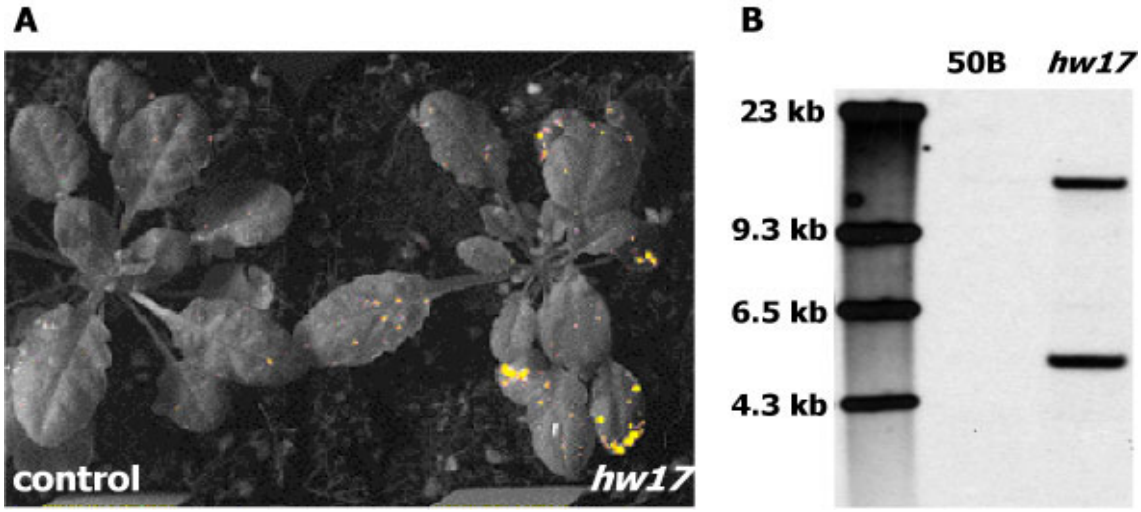


Figure 15: The analysis of the hyper-recombination mutant *hw17*
A. The increased frequency of homologous recombination events of the *hw17* mutant compared with a control plant. **B.** Southern blot analysis of the T1 *hw17* plant. Genomic DNA was digested with *Hin*dIII and the number of T-DNA copies was detected by a probe specific for the *Ampicillin* gene. (For more details, see Supplementary data, page 123. The pictures were kindly provided by O. Fritsch.)

Apart from the increased somatic homologous recombination frequency no growth or developmental phenotypes were observed. The number of rosette leaves, the flowering

time and fertility seemed to be the same as in wild type plants. In contrast, some other recombination-up mutants isolated in the same genetic screen exhibited severe phenotypic alterations [Fritsch, 2004].

2.1.2 Confirmation of the homologous recombination phenotype

In mutant plants isolated in genetic screens using T-DNA transfer by *Agrobacterium tumefaciens* for mutagenesis, the link between integration site and phenotype could often not be established. Concomitant with T-DNA integration secondary mutations may arise in the genome. Therefore, it is necessary to test the linkage between the HR phenotype and the mutagenising T-DNAs. The mutant line *hw17* was backcrossed twice to 50B plants and the co-segregation of the two T-DNA mutations with the HR phenotype was pursued in the backcrossed as well as in selfed population over several generations (Figure 16).

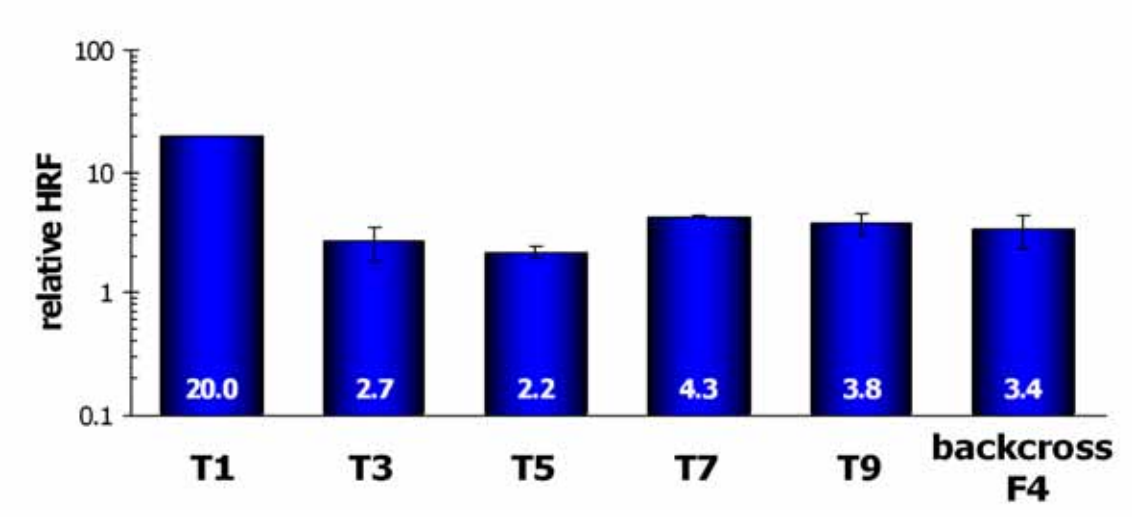


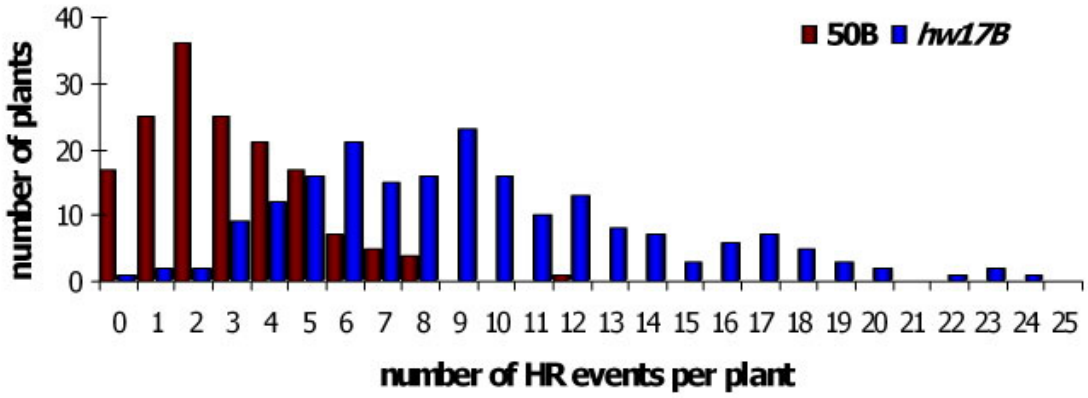
Figure 16: The confirmation of the homologous recombination phenotype
The homologous recombination frequencies (HRF) in *hw17B* populations were compared to those of control plants up to the 9th generation. Plants backcrossed twice to 50B and selected for the *hw17B* locus were also analysed. Error bars indicate the standard deviation of several experimental repetitions.

F4 populations harbouring either of the two integrated T-DNAs were analysed for their recombination frequency. *Hw17A* plants did not reveal any changes in the homologous recombination frequency (data not shown); furthermore, a sulfonamide resistance was not linked to this integration site. In contrast, all *hw17B* mutant plants were found to be sulfonamide resistant, indicating the presence of a functional and presumably full length T-DNA in this locus. For the backcrossed *hw17B* line, the homologous recombination frequency (HRF) was assessed and statistically analysed in hemizygous

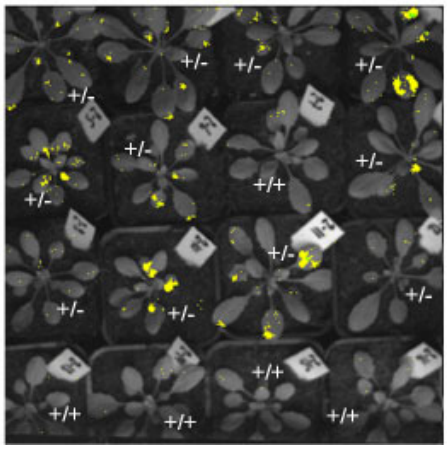
populations of F4 plants. A relative increase of about 3 times was observed in comparison with a wild type population derived from segregating plant of the initial backcross (Figure 16, last lane).

The persistence of the HR phenotype was analysed over several generations. The measured increase of somatic homologous recombination in plants with the *hw17B* locus was consistently observed but varied in the different generations, being 2 to 4 times higher than controls (Figure 16). In *hw17B* plants, the increased HRF phenotype was linked to the T-DNA and was stable over generations. This is on contrast to most other primary mutants obtained from the same screen, the hyper-recombination phenotype of which could not be confirmed in subsequent generations [discussed in: Fritsch, 2004].

A



B



C

Single factor ANOVA:

Groups	Count	Sum	Average	Variance
50B	153	443	2.90	4.52
hw17B	203	1950	9.61	25.55

p-value:	**between groups:**	***2.06E-41***

Figure 17: Statistics of homologous recombination events in individual plants

A. Cumulative distribution of plants with particular number of HR events. **B.** A representative picture showing the HR events of individual plants in the segregating offspring of a hemizygous *hw17B* mutant plant. **C.** Statistical analysis of the distribution of the HR events by single factor ANOVA.

In the previous experiments the homologous recombination frequencies were always assessed in groups of 15 to 30 plants, assuming a standard distribution of the number of HR events per plant. Statistical analysis was done on normalised HR frequencies of these populations obtained from numerous experimental repetitions. Since statistics is only legitimate in populations with standard distribution, the number of HR events was scored for 150 wild type (50B) and for 200 *hw17B* plants and blotted as a cumulative histogram (Figure 17A, B). The peak of the HR events distribution in a 50B population was at 2 events per plant (average: 2.9). For *hw17B* mutant plants the maximum peak was shifted to about 9 events (average: 9.61) (Figure 17A, C). Both graphs resembled the typical standard distribution, except that the variance of HR events in an *hw17B* population was found to be 6 times higher than for control plants. Statistical comparison of the HR events of the *hw17B* and 50B population by single factor ANOVA revealed a highly significant difference (*p*- value 2.06E-41) between the two genotypes.

2.1.3 Molecular characterisation of the *hw17* mutant plants

2.1.3.1 Genomic analysis

The insertion sites of the two mutagenising T-DNAs were isolated by a "plasmid rescue" method [Kiessling *et al.*, 1984]. The *Hin*dIII restricted genomic DNA of *hw17A* and *hw17B* plants were enzymatically circularised and electroporated into a methylation-insensitive *Escherichia coli* strain. Colonies surviving on selective media harboured the bacterial resistance gene, an origin of replication (ori), T-DNA right border (RB) and genomic Arabidopsis DNA. Plasmids, showing the expected *Hin*dIII restriction fragments of 13kb and 5kb for *hw17A* and *hw17B*, respectively, were sequenced using primers in the proximity of the RB (rb-nos#1) as well as of the *Hin*dIII site (M13 reverse). The junction of T-DNA and genomic DNA for the *hw17A* locus was found to consist of repetitive sequences which could not be exactly positioned in the Arabidopsis genome (data not shown). In the *hw17B* locus the T-DNA was integrated into genomic DNA covered by the BAC clone MBM17 (gi: 3869066) which maps to the end of the long arm of chromosome 5. The RB junction was pinpointed to position 20,310 of MBM17 (Figure 18A, B), corresponding to intron sequences of the predicted gene at5g63950 which was annotated as a protein similar to the budding yeast transcription-coupled NER enzyme Rad26.

Since the *hw17A* locus could not be mapped precisely and the HR phenotype was shown to be linked to the *hw17B* locus, the mutation in the latter will hereafter be referred to as *hw17*.

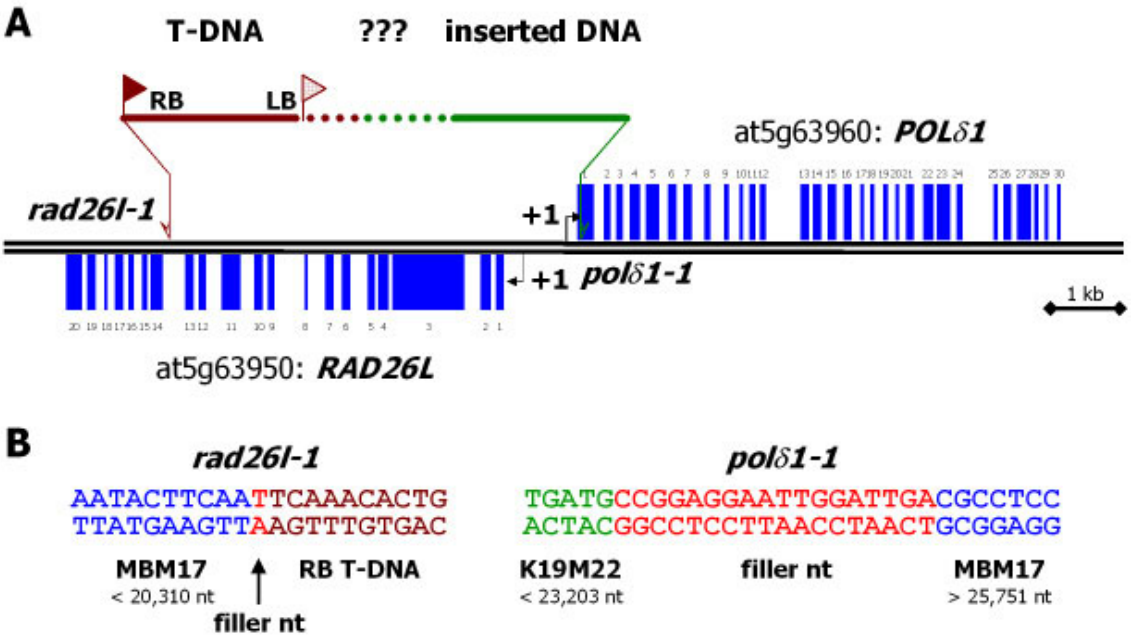


Figure 18: A schematic representation of the mutated *hw17* locus
A. Simplified molecular description of the mutagenised locus, depicting the mutations of two adjacent genes by the integrated T-DNA. **B.** Junction sequences of the *rad26l-1* and *polδ1-1* allele. Blue colour indicates genomic DNA sequences, brown colour stands for T-DNA sequences, green colour shows the ectopic sequences and red colour symbolises filler DNA. +1 marks the transcription start sites of the two genes.

The LB border junction of the T-DNA turned out to be much more difficult to obtain. Extensive insertions, deletions or rearrangements may have happened in the locus during T-DNA integration (for details see Supplementary data, page 123). Figure 18A shows a simplified map of the *hw17* locus with the proven molecular events. A full length T-DNA was found to have integrated but the LB junction with genomic Arabidopsis sequence could not be isolated so far. Furthermore, a stretch of Arabidopsis DNA, which corresponded to sequences of the TAC clone K19M22 (gi: 3449326), was duplicated and reinserted into the genome in the *hw17* locus. This insertion accompanied by 18 nucleotides of filler DNA of unknown origin happened about 6 kb away from the RB integration site, corresponding to position 25,751 on the BAC clone MBM17. The junction was placed 31 nt down-stream of the predicted start codon and thereby interrupting the open reading frame of the gene at5g63960 annotated as the catalytic subunit of the DNA polymerase δ.

In conclusion, the T-DNA integration event in the *hw17* locus created two mutations in genes putatively functioning in DNA metabolism. In both cases the open reading frame was interrupted by the insertion. Mutations in other genes could not be excluded and a molecular link between the mutation in the *RAD26*-like and the *POLδ1* genes could not be made; therefore, they were named *rad26l-1* and *polδ1-1*, respectively.

2.1.3.2 Transcriptional analysis

The activation-tag of the mutagenising T-DNA contains a full Cauliflower Mosaic Virus (CaMV) 35S promoter including strong enhancer sequences. This transcription enhancer element was shown to up-regulate the expression of nearby genes [Weigel *et al.*, 2000]. The CaMV 35S promoter in close proximity to the RB of the T-DNA can alter the expression of the adjacent gene by replacing or enhancing the endogenous promoter and also by producing antisense transcripts leading to an RNAi-based reduction of its steady-state level. Other modes of action may be: ectopic expression of a dominant negative gene product or simply the knock-out of a gene. For the understanding of possible causes of the homologous recombination phenotype, the transcriptional alteration in hemizygous *hw17* mutants was studied by semi-quantitative RT-PCR and by Affymetrix microarray analysis. The microarray technique allows the assessment of genome-wide gene expression by quantification of labelled RNA that hybridises to oligonucleotides on the array covering the 3' regions of virtually all Arabidopsis genes. The RNA steady-state levels of wt and hemizygous *hw17* plants was measured twice in independent experiments and then compared.

The activation-tag of the T-DNA was found to be functional; it strongly boosted the transcription of the *RAD26L* gene 3' of the insertion site (*RAD26L* 3') (Figure 19A). The microarray analysis confirmed this data and a 63 times induction of the steady-state level of *RAD26L* transcripts was measured (Table 8). The steady-state level of full length transcripts of the *RAD26L* gene (*RAD26L* ct) was slightly reduced in mutant plants, indicating that no transcriptional compensation by up-regulating the expression from the homologous chromosomes took place (Figure 19A). In the microarray analysis a 3 fold increase of the transcript level was measured for the *POLδ1* gene, which could also be seen in the RT-PCR reactions, using primers in the 3' region of the gene (*POLδ1* 3') (Table 8 and Figure 19A). In contrast, the level of full length *POLδ1* transcript (*POLδ*1 5'U) was reduced, which suggested that less functional POLδ1 protein may be synthesised in the mutant background. The dominant fashion of the HR induction in *hw17* may be caused by the transcriptional reduction of either - *RAD26L* or

POLδ1 - gene or by a dominant-negative truncated polypeptide encoded by the aberrant transcripts.

Significant transcriptional changes for any nearby genes or for genes located in the duplicated region were not observed in *hw17* mutant plants (Table 8B). In addition, the expression levels of only few genes were changed more than 2.5 fold, of which only the gene at4g05030 passed the statistical significance test (Table 8A). This indicated that the mutations in the *hw17* locus did not lead to substantial general transcriptional alterations and therefore, they may directly be responsible for the increase of the somatic homologous recombination phenotype.

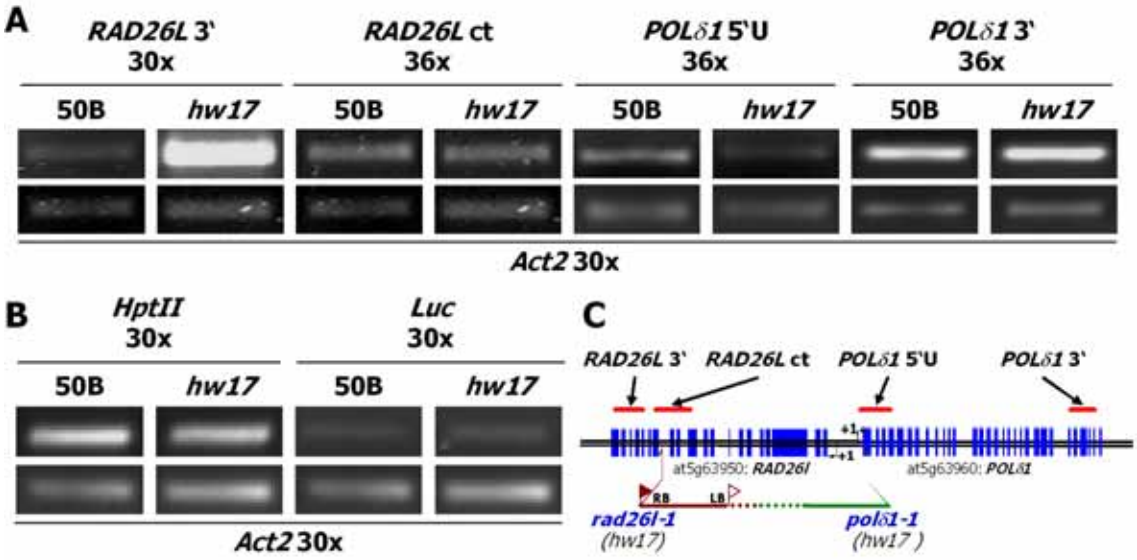


Figure 19: Transcriptional changes in the *hw17* mutants
Detection of expression levels by semi-quantitative RT-PCR for genes in the *hw17* locus (**A**) and in the recombination substrate locus (**B**). The number of PCR cycles and the amplified transcripts by the gene-specific primers are indicated in the top line. 30 PCR cycles amplifying the constitutively expressed *Actin2* gene (*Act2*) served as control and expression standard. **C.** Schematic map of the *hw17* locus: red lines indicate the position of mRNA fragments amplified by the RT-PCR.

Previously it was found that different Arabidopsis substrate lines exhibited varying basal frequencies of somatic recombination [Swoboda *et al.*, 1994; Puchta *et al.*, 1995a]. These observations were explained by influences of the genomic region into which the recombination substrates had been integrated and which are generally termed “position effects”. The chromatin state controls the accessibility of the DNA for repair as well as transcriptional activity [reviewed in: Meijer and Smerdon, 1999; Lusser, 2002]. RNA steady-state levels in the 50B substrate locus were measured by semi-quantitative RT-PCR in order to exclude the possibility that local chromatin changes in *hw17* mutants led to increased expression in the recombination substrate

locus of 50B plants, which could also results in a moderate increase of HR. This substrate locus contains two transgenes driven by strong promoters: a truncated *Luciferase* (*Luc*) gene and the *Hygromycin resistance* (*HptII*) gene (Figure 14, page 61). The transcription levels of both the *Luc* and the *HptII* gene were found to be only slightly affected in *hw17* plants compared to the line 50B, suggesting a direct causal effect of the *hw17* mutations on HR (Figure 19B).

A

	fold change	gene	annotation	statistically significant
up	63.22	AT5G63950	RAD26-like protein	*
	7.476	AT4G05030	similar to farnesylated protein atFP4	*
	5.39	AT5G51900	cytochrome P450 like protein	
	2.90	AT5G63960	DNA polymerase type III, catalytic subunit	
	2.78	AT4G10950	proline-rich protein	
	2.76	AT2G03580	hypothetical protein	
	2.63	AT2G14610	PR1-like protein	
	2.51	AT1G33090	hypothetical protein	
down	3.51	AT4G15210	beta-amylase	
	3.07	AT3G21720	putative isocitrate lyase	
	2.98	AT4G33720	pathogenesis-related protein 1, precursor	
	2.52	AT4G15190	hypothetical protein	

B

fold change		gene	annotation	statistically significant
down	1.05	AT5G63890	histidinol dehydrogenase	
up	1.92	AT5G63900	PHD-type zinc finger protein	
down	1.28	AT5G63905	unknown protein	
down	1.03	AT5G63910	unknown protein	
down	1.10	AT5G63920	DNA topoisomerase III	
up	1.10	AT5G63930	receptor-like protein kinase	
down	1.05	AT5G63940	protein kinase	
up	63.22	AT5G63950	RAD26-like protein	
up	2.90	AT5G63960	DNA polymerase type III, catalytic subunit	
up	1.08	AT5G63970	unknown protein, copine related	
down	1.03	AT5G63980	3(2),5-bisphosphate nucleotidase	

Table 8: Affymetrix microarray analysis of the *hw17* mutant
A. List of genes with more than 2.5 fold altered expression levels in the *hw17* mutant compared to control plants. **B.** Transcriptional changes in the *hw17* locus. Asterisks mark transcriptional changes, which pass the ANOVA significance test.

2.1.4 A deleterious mutation in the *hw17* locus

No homozygous mutants for the *hw17* locus could be obtained, suggesting that an essential gene is knocked-out. In fact, the sulfonamide resistance and sensitivity in the offspring of a hemizygous mutant plant was segregating in a 2:1 ratio. The segregation was analysed in a population of about 300 seedlings, yielding a significant difference to

the expected 3:1 Mendelian ratio for a recessive trait (χ^2: *p*-value=0.004) and resembling more the 2:1 ratio expected for a recessive mutation in an essential gene (χ^2: *p*-value=0.70). This segregation rate typically indicates a deleterious effect of a homozygous mutation in zygotes.

The visual inspection of siliques of hemizygous plants revealed an atypical high number of late aborting seeds (Figure 20A, B). The presence of pale green seeds without a functioning embryo and of desiccating and brown seeds indicated differences in the timing of seed degeneration. Statistical analysis of siliques originating from different independent mutant plants confirmed that about 25% of the seeds aborted, which significantly exceeded the number observed in siliques of wt plants (single factor ANOVA, *p*-value: 1.49E-09) (Figure 20C). This number correlated well with the absence of homozygous mutations in the progeny of a hemizygous *hw17* plant.

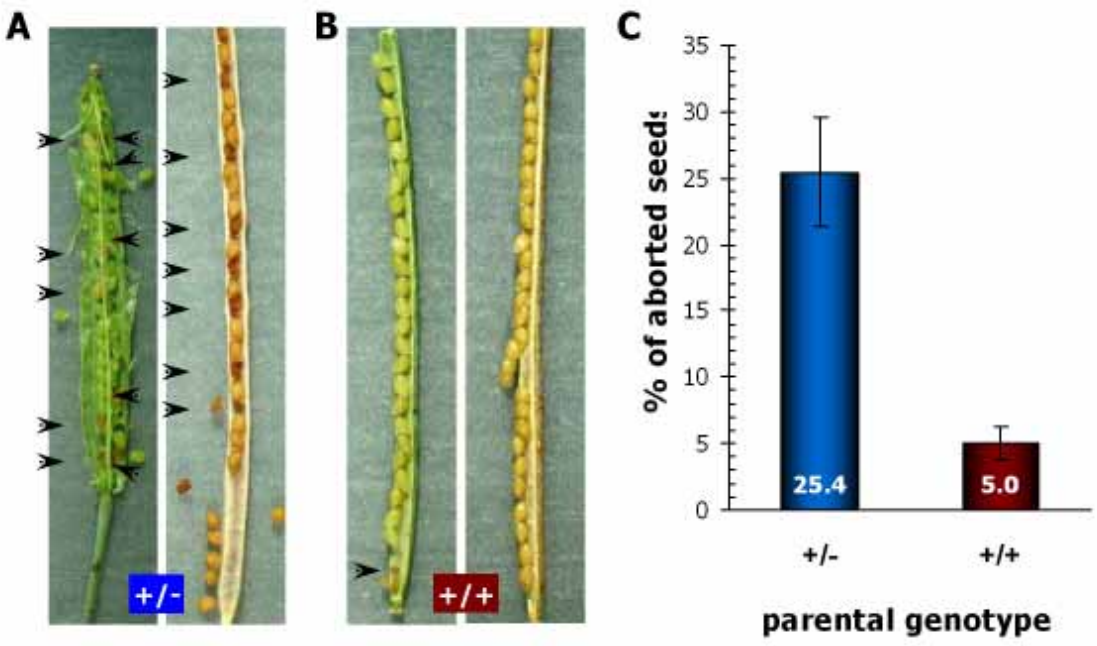


Figure 20: The late seed abortion in selfed hemizygous parental *hw17* mutants
A. *hw17* mutant siliques showing aborted seeds; first pale (left) and later desiccated and dark brown. **B.** Siliques of wildtype plants at comparable stages. **C.** Percentage of late aborted seeds in the siliques of hemizygous *hw17* mutants and segregating wildtype plants.

Early embryo development was examined microscopically, using a whole mounting and tissue clearing method. In a wildtype background fertilized ovules in a given silique developed synchronously. In the offspring of a hemizygous *hw17* mutant plant 25% of the fertilised seeds were found to abort. Indeed, a similar proportion of atypical embryo development was observed. Already at an early stage (Figure 21A) the growth

of the putative *hw17* -/- embryos was severely affected; the zygotes stopped dividing after the first unequal cell division that gives rise to suspensor and embryonic structures.

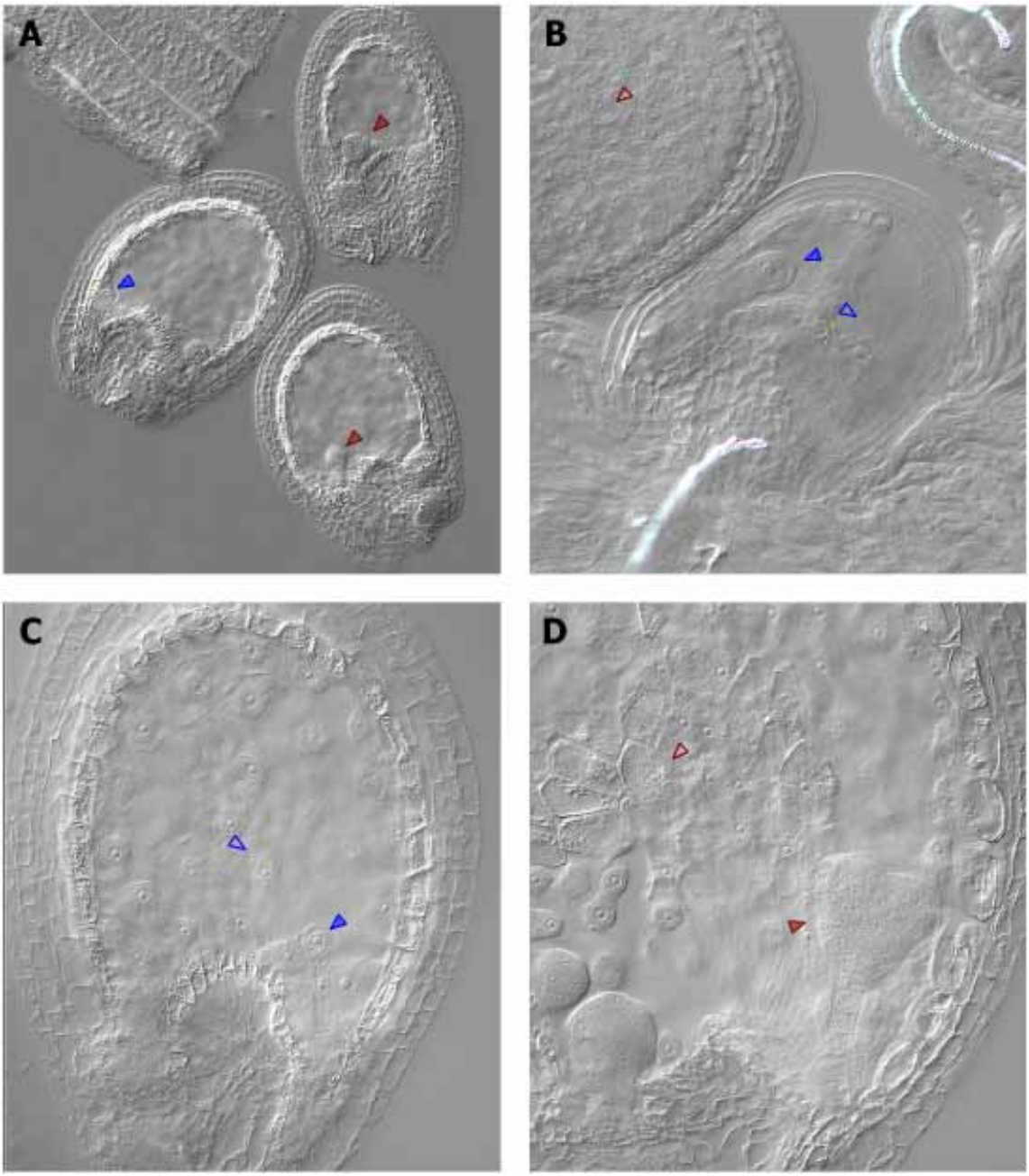


Figure 21: The arrested development of a homozygous *hw17* embryo
A. Early Arabidopsis embryos ranging from 2-cell to globular developmental stages observed in the same silique of a selfed hemizygous *hw17* plant. **B.** Later stages of seed development in which wt endosperm is cellularised and starts accumulating starch and wt embryos are in late heart/early torpedo stage. **C.** Putative *hw17* -/- structure in which the embryo was arrested after the first unequal division and the endosperm is multicellular. **D.** Wildtype ovule at a comparable stage of seed development with a heart shaped embryo and clearly cellularised endosperm. Blue arrowheads, mutant structures; brown arrowheads, wt structures; filled shapes, embryo; empty shapes, endosperm.

These arrested embryos persisted also to later stages of seed development (Figure 21B, C). In contrast, the triploid endosperm was found to exhibit ambiguous developmental phenotypes. Some of the endosperms developed normally into a multi-nuclear and subsequently clearly cellularised structure similar to wt (Figure 21C, D); in other seeds such structures were not found (Figure 21B). Although the developmental phenotypes of these embryos observed in the offspring of a selfed, hemizygous *hw17* plant could not be correlated with a homozygous mutation by molecular means, they fitted in well with the previously observed distorted segregation and the seed abortion. These findings suggest a deleterious effect of the homozygous *hw17* locus on cell division and thus on embryo development.

2.2 Cloning of the candidate genes

The molecular analysis of *hw17* mutants revealed two main candidate genes, mutations in which may have caused its HR phenotype. The cDNAs of both of them were amplified and cloned, using RT-PCR techniques. For the *RAD26L* gene a cDNA of 3,578 bp was cloned, containing an ORF of 3,270 nt, 100 nt of 5'-untranslated region (UTR) and 208 nt of 3'-UTR (Figure 22; for the complete sequence see Appendix, page X). This sequence corresponded to the RIKEN full length cDNA clone RAFL09-10-H17 deposited in the public database (gi: 18087572). The alignment of the cDNA with the genomic sequence covered about 6.7 kb of its complementary strand. The *RAD26L* gene consisted of 20 exons and 19 introns, of which all splicing sites corresponded to Arabidopsis consensus sequences [Hebsgaard *et al.*, 1996].

For the isolation of the full length cDNA of the *POLδ1* gene, the circularisation RT-PCR (cRT-PCR) method was used to amplify the 5'- and 3'- extremities. Two distinct cDNA sequences for the *POLδ1* gene were obtained with a length of 3,537 and 3,617 bp, respectively (Figure 22; for the complete sequences see Appendix, page VII). Both of them contained 83 nt 5'-UTR sequence, used the same polyadenylisation signal and their ORFs also started at the same codon. The difference between the two cDNA sequences originated from the lack of splicing of the 25th intron, which resulted in an 80 nt longer cDNA for the splice variant. In this longer transcript, a premature stop codon located in the unspliced intron leads to the shortening of the POLδ1 encoding ORF from 3,285 nt to 3,141 nt. The question whether both of these cDNAs have a biological significance remains to be answered. The coding region of the *POLδ1* gene spanned about 7 kb genomic sequence of the coding strand, consisting of 30 exons and 29 introns (see Figure 22B).

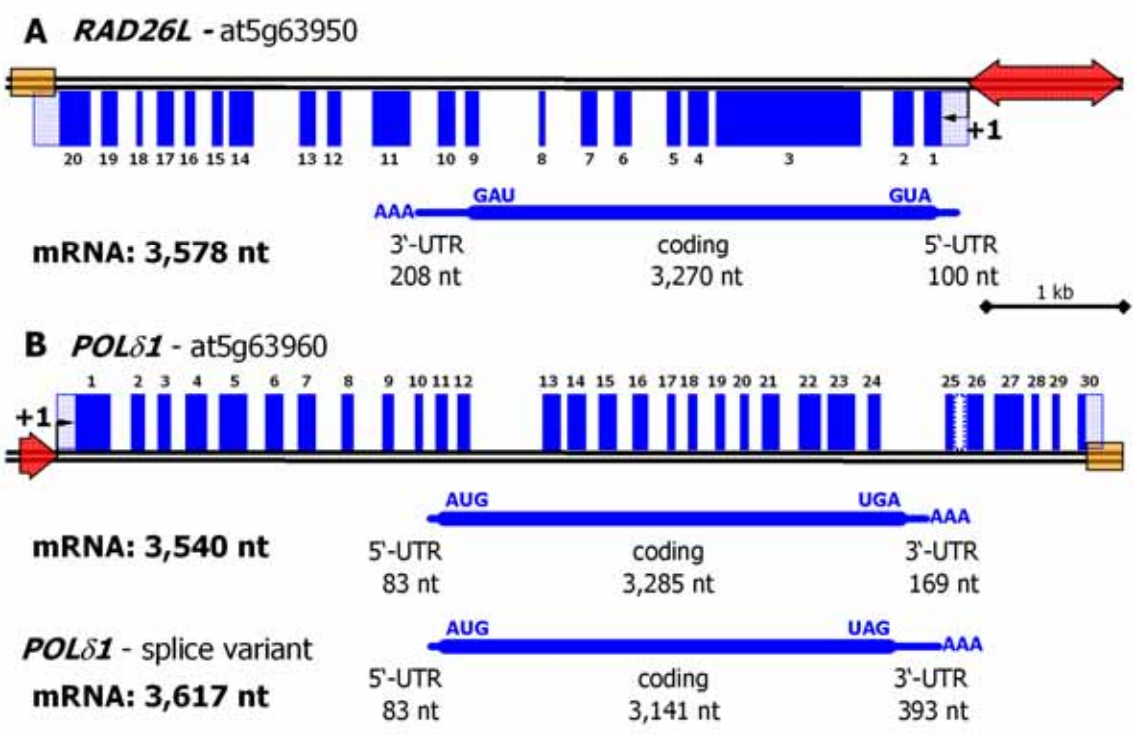


Figure 22: The organisation of the *RAD26L* and *POLδ1* genes
The two genes are encoded on the sense (**B**, *POLδ1*) and the complementary strand (**A**, *RAD26L*), separated by a 1.1 kb region containing the promoter sequences for both (red arrow). The length and the positions of the exons on the genomic DNA (black double line) are indicated as blue boxes. The mRNAs corresponding to the cloned cDNAs are depicted as blue lines. Light blue boxes symbolise sequences corresponding to the untranslated region of the mRNA. +1 marks the transcription start and orange boxes the putative terminator region.

2.3 Genetic dissection of the *hw17* locus

2.3.1 Homologous recombination phenotypes of allelic mutants

A complex genome rearrangement and mutations in at least two genes were found in the hyper-recombination mutant *hw17*. Genetic and molecular biological strategies were applied in order to pinpoint the mutation responsible for the HR phenotype. Public databases of Arabidopsis T-DNA knock-out (KO) lines were searched for mapped insertions in the *POLδ1* and the *RAD26L* gene. Interesting enough, many KO lines with T-DNA insertions in *RAD26L* could be found but only two of them in the *POLδ1* gene. Two lines for each gene were ordered from the T-DNA insertion collection of the SALK institute and characterised on a molecular level (for details see Supplementary data, page 123). In the SALK_053085 line, the T-DNA was integrated into the 5'-UTR of the *POLδ1* gene, completely depleting the wild type transcript but slightly increasing the steady-state level of an aberrant mRNA that may encode for a functional POLδ1 protein

(see Supplementary data, page 132). This allele was named *polδ1-2*. In the SALK_030272 line, termed *polδ1-3* allele, the ORF was found to be interrupted (Figure 23). Two *rad26l* alleles were characterised and named *rad26l-2* and *rad26l-3* for the lines SALK_050793 and SALK_007071, respectively. In both of them the T-DNA is likely to interrupt the ORF of the *RAD26L* gene: in *rad26l-2* by a deletion of the exons 12 and 13 and in *rad26l-3* by its integration into the 3rd exon (Figure 23). Homozygous mutants could be obtained for all KO lines with the exception of *polδ1-3*, suggesting a causal link between mutations in the *POLδ1* gene and the arrest of embryo growth.

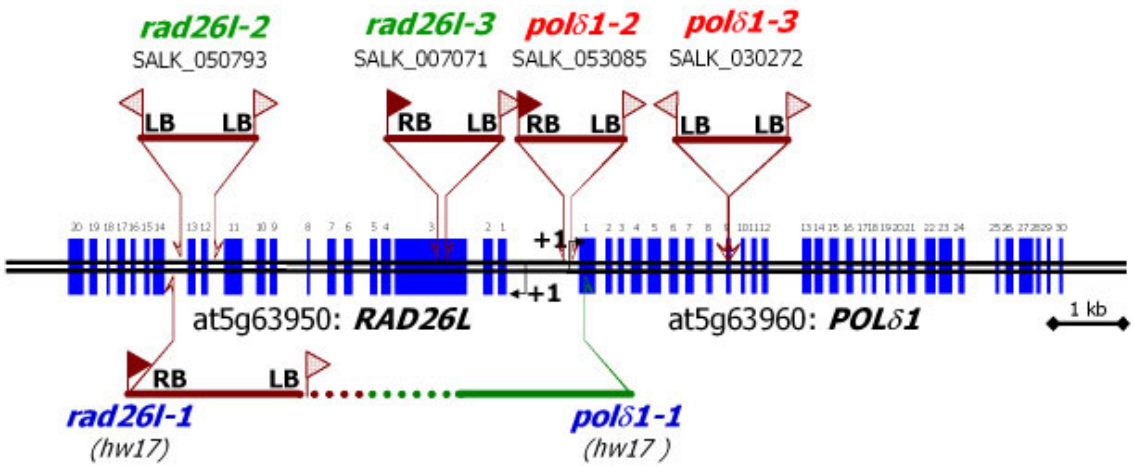


Figure 23: A map of the allelic mutations for genes in the *hw17* locus
T-DNA mutagenised lines from the SALK Institute were molecularly analysed and placed accordingly in a schematic map of the *hw17* locus. Half arrow heads indicate the positions of T-DNA border junctions within the respective genes. +1 marks the transcription start sites; blue boxes indicate exons.

All four KO lines were crossed into the intra-molecular recombination reporter lines 50B and 1445 (see page 60). Plants homozygous for the substrate locus and for the *polδ1* or the *rad26l* alleles were isolated (hemizygous for *polδ1-3*). F4 mutant families were analysed for their somatic HRFs and compared with control populations, segregating wt plants of the crosses. Homozygous *polδ1-2* plants revealed a 2.3 times higher HR frequency, whereas hemizygous plants did not yield any change (Figure 24). In contrast, hemizygous *polδ1-3* plants showed a more pronounced increase of the HRF. In none of the *rad26l* alleles a significant change of the homologous recombination frequency was observed. These findings clearly suggest that mutations in the *POLδ1* and not in the *RAD26L* gene influence the level of somatic homologous recombination.

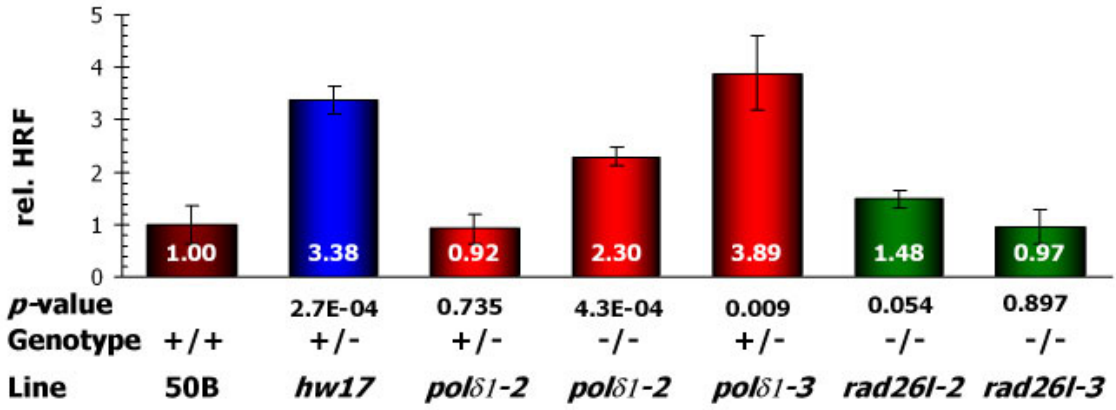


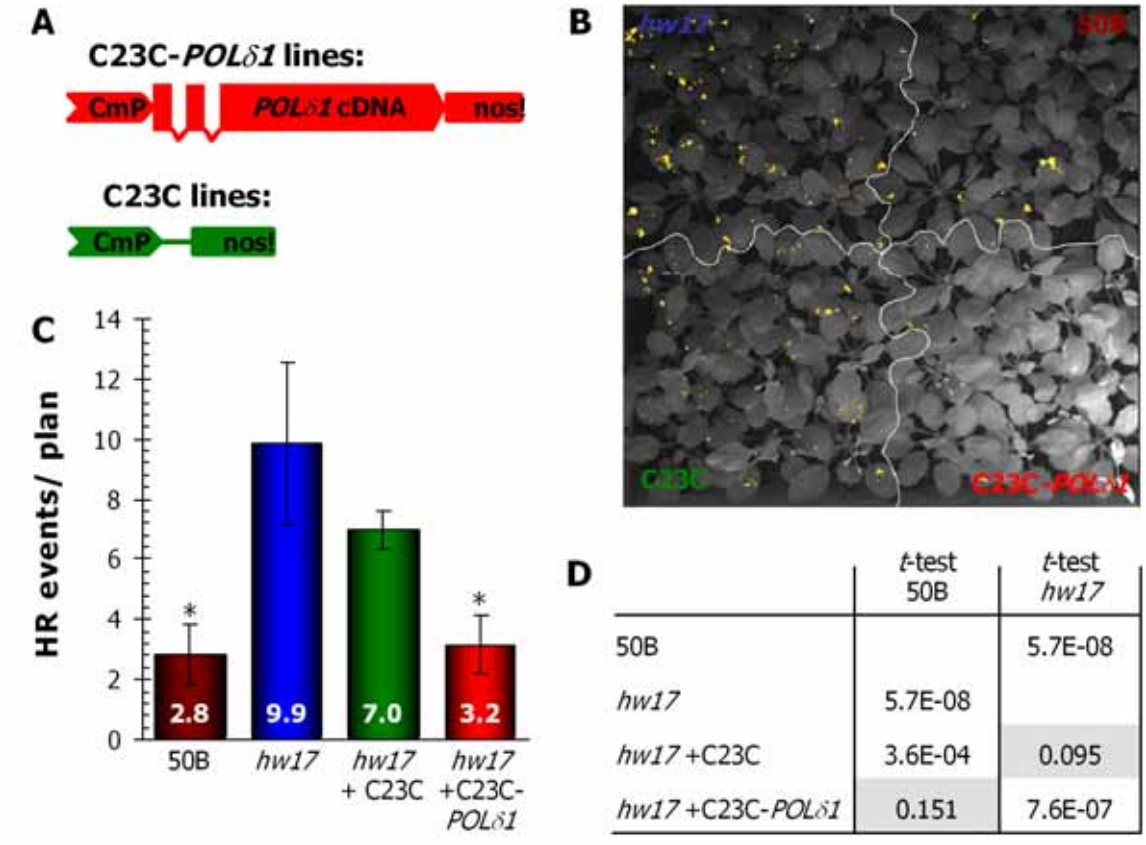
Figure 24: The recombination frequencies of *polδ1* and *rad26l* alleles
F4 mutant families with the intra-molecular recombination substrate from line 50B were scored for somatic HR events and frequencies relative to their controls were calculated. The *p*-value indicates the significance of the differences between the respective mutant line and the control, analysed by single factor ANOVA. Wt (+) and knock-out (-) indicate the genotype of the haploid genome in the respective gene. Error bars indicate standard errors of several independent experiments.

2.3.2 Complementation and reconstruction of the HR phenotype

The analysis of the HR frequency in mutant alleles of both candidate genes suggested that the mutation in *POLδ1* caused the hyper-recombination phenotype observed in the *hw17* mutant. A complementation analysis would further substantiate this finding; the ectopic expression of either of the cDNAs in the *hw17* background was analysed for their suppression of the HR level phenotype. In addition, this complementation experiment was thought to elucidate mechanistic aspects of the dominant nature of the mutations.

For the complementation with *POLδ1* a chimeric gene of genomic and cDNA sequences was constructed, including the two first endogenous introns of the gene (Appendix, page IX). This was required since plasmids that only contained the cDNA severely interfered with growth of *E.coli* and surviving colonies were found to accumulate mutations in *POLδ1* (data not shown). Presumably, basal expression of the Arabidopsis DNA polymerase δ was disturbing bacterial DNA replication or the exonuclease domain of POLδ1 may have attacked the genome of the host. The chimeric *POLδ1* gene was cloned into the binary vector pC23C downstream of a strong viral promoter [Stavolone *et al.*, 2003] (Plasmid 2, page XIV and Figure 25A). Hemizygous *hw17* plants were transformed by the floral dipping method with *A.tumefaciens* containing the binary vector with the *POLδ1* cDNA or the control construct.

T1 transformants were selected for the complementing T-DNA and genotyped by PCR for the presence of the *hw17* locus. In the T2 generation hemizygous *hw17* plants of four control and ten *POLδ1*-complemented families were obtained by double-selection. In three independent experiments a suppression of the hyper-recombination phenotype of *hw17* was found for all families transformed with the *POLδ1* cDNA construct except for family 14 (Figure 25B, Figure 26A).



D

	t-test 50B	*t*-test *hw17*
50B		5.7E-08
hw17	5.7E-08	
hw17 +C23C	3.6E-04	0.095
hw17 +C23C-*POLδ1*	0.151	7.6E-07

Figure 25: Summary of *POLδ1*-complemented *hw17* lines
A. Schematic representation of the constructs used for the complementation experiment: *POLδ1* cDNA, chimeric genomic and cDNA sequence of *POLδ1*. Cmp, promoter region of the cestrum yellow leaf curling virus [Stavolone *et al.*, 2003]. nos!, terminator sequence of the *A.tumefaciens Nopaline synthase* gene. **B.** A representative picture of the complementation experiment showing the HR events in 50B and *hw17* population and in two T2 families complemented either by the vector control (C23C) or by the cDNA of *POLδ1*. **C.** HR frequencies of 9 independent *POLδ1*-complemented T2 families and of 4 lines transformed with the vector control compared to 50B and to *hw17* plants. Error bars indicate the standard deviations and asterisks mark significant differences to *hw17*. **D.** Statistical analysis of the complementation experiment: *t*-test of the HR means comparing 50B and *hw17* populations with the complemented families.

The analysis of the ectopic expression of *POLδ1* by RT-PCR correlated well with the suppression of the mutant phenotype (Figure 26A, B). For instance no ectopic *POLδ1* transcript could be detected in family 14 in which no suppression of the HR

enhancement was observed. Plants transformed with the vector control revealed a slight reduction of their HR frequencies, possibly caused by promoter-triggered co-suppression of the substrate locus. However, no significant change of the transcriptional activity of the *HptII* gene in the 50B locus could be found.

The data of the nine complemented and the four control families was combined and statistically analysed (Figure 25C, D). Ectopic expression of the *POLδ1* cDNA reduced the number of detected HR events in an *hw17* background from 9.9 to 3.2, which was not significantly different from the basal HR frequency of 50B plants (*p*-value: 0.151). In contrast, the HR frequencies of *hw17* plants transformed with the vector control dropped from 9.9 to 7 events, which were found to be not significantly different to the ones of *hw17* mutant plants (*p*-value: 0.095).

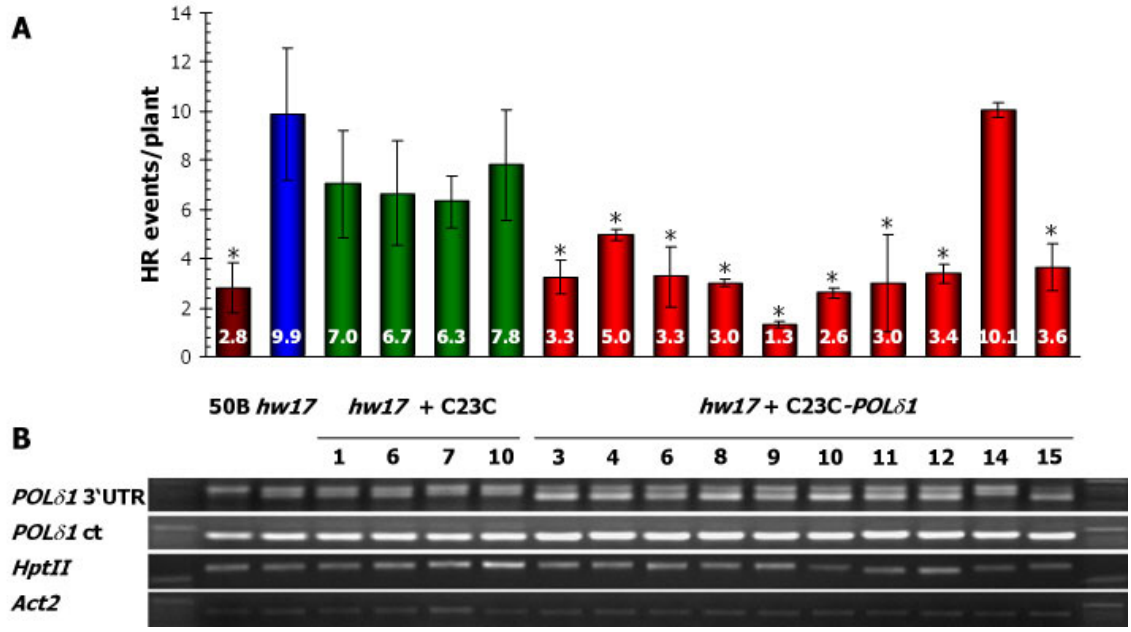


Figure 26: The complementation of the *hw17* mutation by the cDNA of *POLδ1*

A. T2 populations of independent transformants were analysed for the effect of ectopic expression of the *POLδ1* cDNA (red) or of a vector control (green) on the HR frequency in an *hw17* mutant background. Error bars indicate standard deviation of 3-5 independent experiments and asterisks mark significant suppression of the enhanced HRF. **B**. RT-PCR analysis of the complemented lines. 1st row: diagnostic PCR for the 3'UTR of native and ectopic *POLδ1* transcripts, wt mRNA 400 bp, alternative polyA site in *polδ1* mutants 350 bp, ectopic *POLδ1* mRNA 250 bp, 36 PCR cycles. 2nd row: diagnostic PCR in the central region of the *POLδ1* mRNA detecting all transcripts, 36 PCR cycles. 3rd row: control PCR detecting the expression of the *HptII* gene in the 50B locus, 30 PCR cycles. 4th row: RT-PCR control detecting the expression of *Actin2* gene (*Act2*), 30 PCR cycles.

The outcome of the complementation experiment strongly supports the previous findings that it was the mutation in the gene coding for the catalytic subunit of the

DNA polymerase δ causing the hyper-recombination phenotype of the *hw17* mutant. Furthermore, the dominance of this mutation can be suppressed by the ectopic expression of the *POLδ1* cDNA. This favours the hypothesis that the reduced level of full length *POLδ1* transcript was causal for the hyper-recombination phenotype of *hw17*. In contrast, complementation with the *RAD26L* cDNA did not yield any difference (data not shown).

2.3.3 Complementation of homozygous lethality

The genotyping of the allelic mutants in both candidate genes already indicated that the mutation in *POLδ1* gene was responsible for the seed abortion and distorted segregation phenotype of *hw17*. In contrast to both *rad26l* alleles, no homozygous mutations could be obtained in the *polδ1-3* mutant. Furthermore, studies of *S.cerevisiae Pol3*, which is the gene coding for the orthologue of the Arabidopsis POLδ1, suggested that the Pol δ complex is essential for DNA replication [Sitney *et al.*, 1989; Budd and Campbell, 1993; Chanet and Heude, 2003].

In the T2 generation the segregation of the *hw17* locus in the complemented families was analysed (see above) in order to elucidate the capacity of the *POLδ1* cDNA to restore the Mendelian 3:1 segregation. For some of the analysed lines, the sulfonamide resistance marker of the *hw17* locus resembled this expected segregation rate (for details see Supplementary data, page 125). Statistically relevant segregation analyses of multiple loci would require a large number of plants, the restoration of the 3:1 segregation was therefore assessed by molecular means. Plants of segregating T2 families were selected *in vitro* for the *hw17* mutation and also for the complementing T-DNA and subsequently they were analysed for the zygousity of the *hw17* locus. As expected, all genotyped plants complemented with the vector control yielded the two PCR bands representing the mutated and the wt gene (Figure 27A, blue boxes). In contrast, T2 families that ectopically express the *POLδ1* cDNA revealed homozygous *polδ1-1* mutations in about one third of the analysed plants (Figure 27B, red boxes). These findings clearly show that the mutation in the *POLδ1* gene was the cause for the distorted segregation, the arrest of embryonic development and late seed abortion.

Driving the expression of *POLδ1* by a viral promoter was not sufficient to overcome the deleterious effect of the *polδ1-1* mutation in all lines. In addition, the growth and the morphogenesis of homozygous *hw17* mutants were severely affected (Figure 27B, C). Germinating homozygous seedlings were phenotypically normal but growth inhibition became more obvious with progressing development. The formation

and the number of rosette leaves appeared to be similar to wt or hemizygous *hw17* plants but they failed to expand, which resulted in a dwarf phenotype. Occasionally, shoots and flowers were formed, even producing a few seeds. In contrast to the above ground part, the growth of the roots was not significantly altered.

It can be concluded that the mutation in the *POLδ1* gene was responsible for the increased homologous recombination frequency and also for the phenotypes related to the failure of proper cellular division. In addition, the suppression of the hyper-recombination phenotype by the *POLδ1* cDNA could not be correlated with a complementation of the division impairment in some lines. This suggests that POLδ1 has a function of in at least two independent pathways or mechanisms.

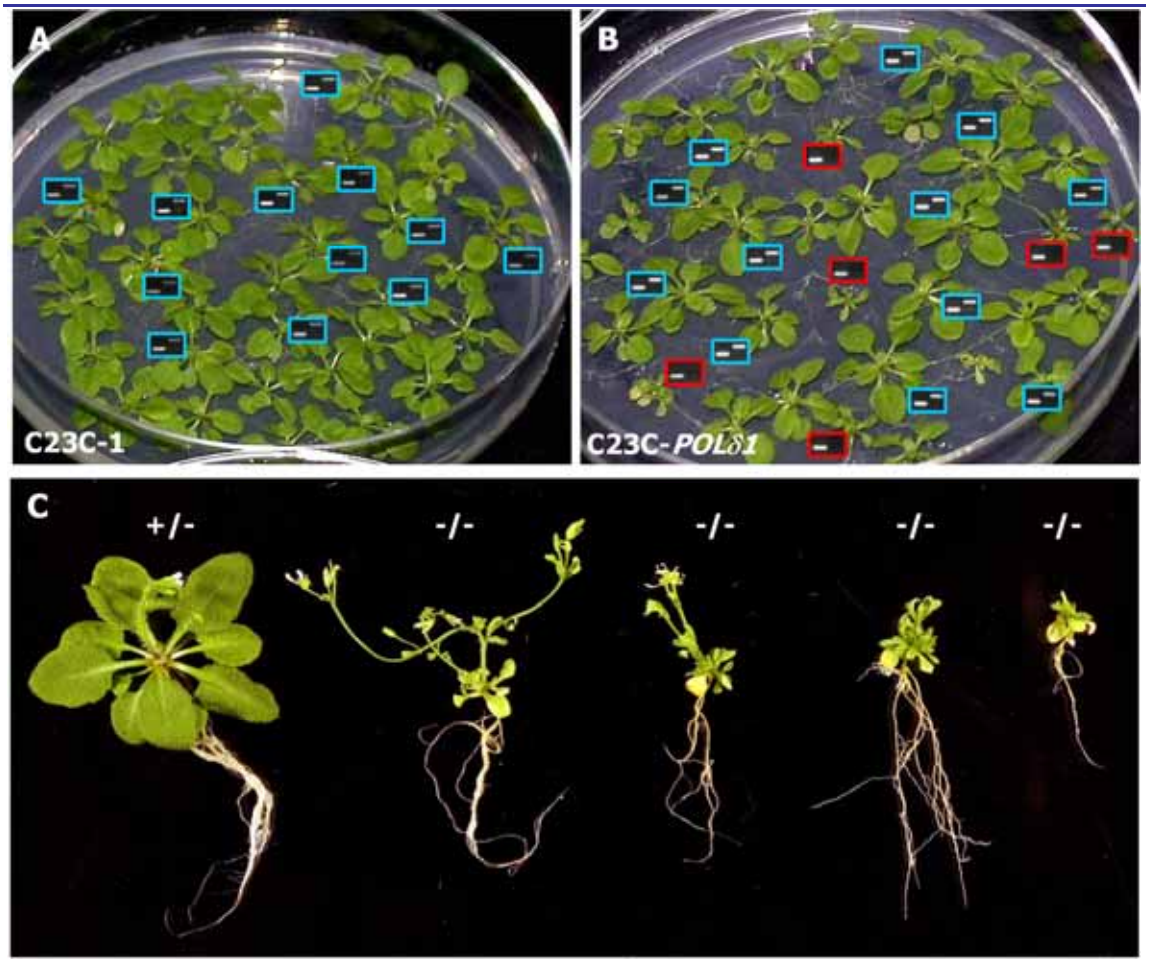


Figure 27: The homozygous lethality in *POLδ1* cDNA-complemented lines
Genotyping was done by PCR specifically detecting the *polδ1-1* mutation (left band) or the wt gene (right band). Hemizygous and homozygous *hw17* plants are indicated by blue and red boxes, respectively. Developmental phenotypes and genotyping in a population of representative T2 families selected for the *hw17* locus and the presence of the complementation control vector (**A**) or the ectopic *POLδ1* cDNA (**B**). Growth phenotypes of homozygous *hw17* plants compared to a hemizygous one (**C**).

2.4 The Arabidopsis DNA polymerase δ and its function

2.4.1 The catalytic subunit of the DNA polymerase δ

The translated cDNA of the Arabidopsis *POLδ1* gene was blasted against public database entries and thereby yielded more than 100 significant hits ranging from viral, bacterial to higher eukaryotic proteins. They were all annotated as DNA-directed DNA polymerases or their catalytic subunits (cs). Most of them belonged to the DNA polymerase family B, members of which are defined by sequence homology, by conserved motifs and by structural similarities [Braithwaite and Ito, 1993]. The catalytic subunits of the three replicative DNA polymerases α, δ and ε belong to this family, all containing 6 highly conserved regions termed I-VI [reviewed in: Joyce and Steitz, 1994; Brautigam and Steitz, 1998]. Significant similarities were also found to the catalytic subunit of the polymerase ζ, which was recently moved into the Y families due to its biological function as error-prone translesion polymerase [Ohmori *et al.*, 2001].

Eukaryotic proteins which showed significant similarities to the Arabidopsis POLδ1 were chosen and their amino acid sequences were aligned by the ClustalW algorithm. Furthermore, their phylogenetic relation was analysed based on these alignments by the bootstrap method. All aligned proteins were found to be described as the catalytic subunit of the multi-subunit DNA polymerase holoenzymes α, δ, ε or ζ, clearly forming four distinct clusters (Figure 28A). Distinct sub-clusters for the mammalian, plant or yeast homologues were observed, whereas the *D.melanogaster* and *C.elegans* homologues mostly represented their own branches. These findings correlated with reported evolutionary relation of these species. Arabidopsis POLδ1 clustered with the predicted or described homologues of the catalytic subunits of Polδ, which proposed an analogous biological function in genome replication and DNA repair. In comparison to the other two replication polymerase families, the short branches of the $Pol\delta_{cs}$ cluster indicated a higher degree of sequence conservation between the different members. More detailed analysis of the sequence composition revealed that the eukaryotic POLδ1 homologues shared many highly conserved motifs and domain arrangement (Figure 28B). They also exhibited an astonishingly high level of sequence similarities (Table 9; Appendix, page XI). More than 40% identical and 60% similar amino acids were found for all compared eukaryotic proteins. Mammalian homologues shared about 90% and more 95% identical and similar residues, respectively. Slightly less conservation was

found within the plant homologues but 75% and 85% of the amino acids still were identical and similar.

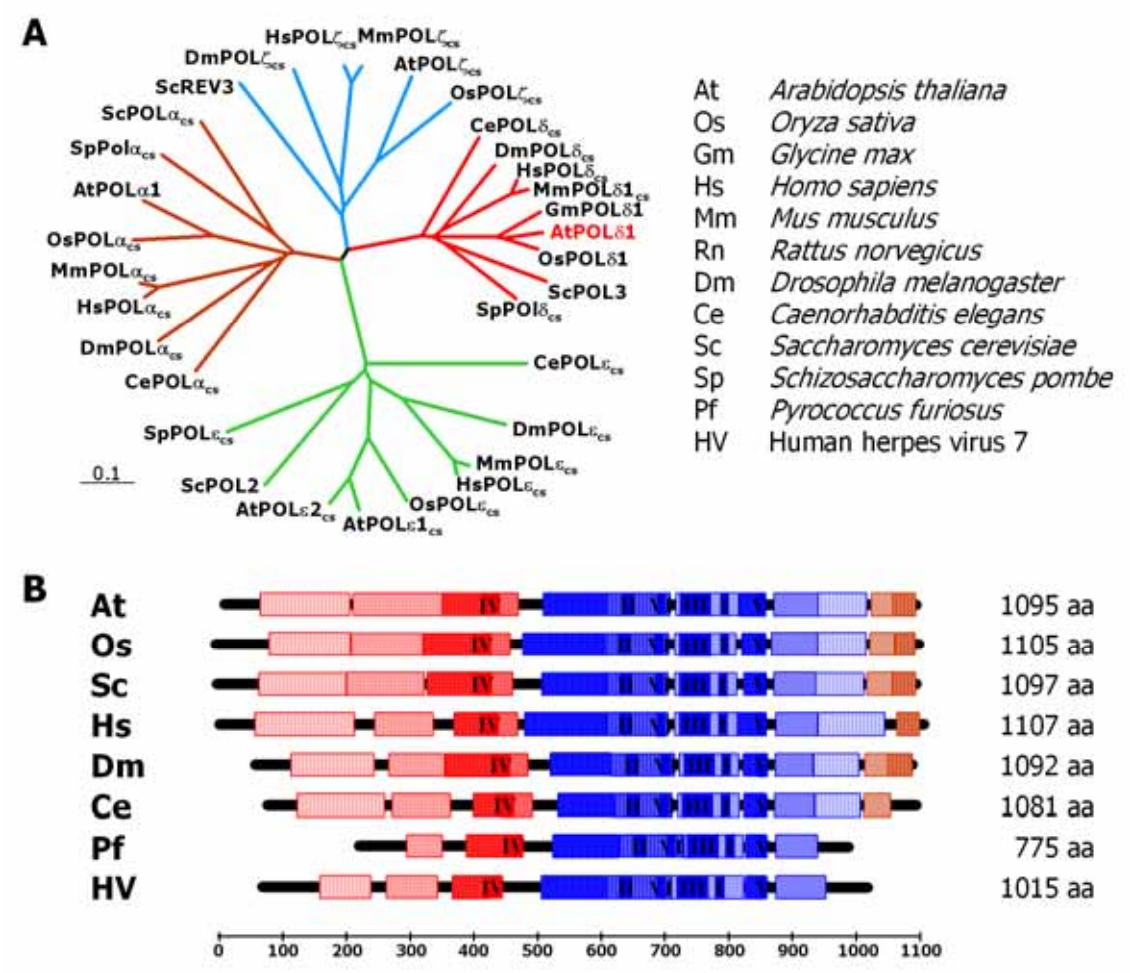


Figure 28: The catalytic subunit of the eukaryotic DNA polymerase δ
A. Phylogenetic relationship of the Arabidopsis POLδ1 protein with other catalytic subunits of eukaryotic DNA polymerase complexes: Polδ (red), Polα (brown), Polε (green) and Polζ (blue). **B.** Automated search for conserved sequences in selected eukaryotic Polδ_{cs} as well as a viral and bacterial DNA polymerase. Red boxes belong to the exonuclease and blue ones to the catalytic domain. Brown boxes indicate other conserved domains. Numbered boxes indicate the six conserved regions of B-type DNA Pols.

The amino acid sequence of Arabidopsis POLδ1 was examined for frequently found protein domains by a bioinformatic analysis. This automated domain analysis confirmed the high level of conservation between the eukaryotic catalytic subunits of Polδ and even between DNA polymerases of bacteria or viruses (Figure 28B). All eukaryotic Polδ_{cs} exhibited significant sequence homology in all regions which constitute the typical arrangement of the exonuclease and the catalytic domains. In addition, the similar spacing of the domains indicated a conserved 3-dimensonal structure that resembles the human right hand with palm, thumb and fingers [Brautigam and Steitz,

1998]. A particular high degree of sequence conservation was assigned to the DNA polymerase B family-specific regions I-VI (compare Appendix, page XI): the -YGDTDS- motif of region I forms the catalytic centre and the dNTP binding site contains the -DxxSLYPS- motif in region II [Hübscher *et al.*, 2002]. Poor sequence conservation was found in the N-terminal region of the Polδ_{cs}, although some motifs such as a putative nuclear targeting signal appeared to be present. Similarly, less sequence homology between POLδ1 and the other Pols was observed at the C-terminus but a conserved bipartite zinc-finger domain could be identified, which may be involved in the interaction with the DNA helix or with other proteins.

S\I	At	Os	Gm	Hs	Mm	Rn	Ce	Dm	Sc	Sp	Ca
At	-	0.797	0.738	0.511	0.513	0.512	0.437	0.497	0.458	0.518	0.506
Os	0.903	-	0.748	0.519	0.517	0.519	0.436	0.508	0.464	0.524	0.500
Gm	0.838	0.845	-	0.474	0.474	0.475	0.416	0.474	0.430	0.474	0.471
Hs	0.701	0.713	0.665	-	0.894	0.897	0.462	0.575	0.445	0.494	0.494
Mm	0.704	0.715	0.669	0.954	-	0.967	0.464	0.571	0.439	0.493	0.492
Rn	0.704	0.714	0.671	0.959	0.984	-	0.461	0.572	0.439	0.491	0.492
Ce	0.644	0.645	0.614	0.687	0.688	0.690	-	0.526	0.410	0.436	0.444
Dm	0.681	0.692	0.656	0.764	0.762	0.767	0.715	-	0.464	0.497	0.501
Sc	0.664	0.674	0.634	0.660	0.668	0.668	0.617	0.653	-	0.520	0.589
Sp	0.697	0.715	0.665	0.719	0.717	0.718	0.642	0.687	0.726	-	0.572
Ca	0.691	0.701	0.660	0.686	0.686	0.684	0.645	0.672	0.749	0.745	-

Table 9: Homologies of the eukaryotic catalytic subunits of the DNA Polδ
Similar (S) and identical (I) amino acid residues of chosen eukaryotic homologues of the Polδ_{cs} (At), aligned by the ClustalW algorithm and analysed with the DAYHOFF matrix for amino acid conservation. The numbers represented the ratio of S or I amino acids and the full sequence. Os, *Oryza sativa*, rice; Gm, *Glycine max*, soybean; Hs, *Homo sapiens*, man; Mm, *Mus musculus*, mouse; Rn, *Rattus norvegicus*, rat; Ce, *Caenorhabditis elegans*, worm; Dm, *Drosophila melanogaster*, fruit fly; Sc, *Saccharomyces cerevisiae*, budding yeast; Sp, *Schizosaccharomyces pombe*, fission yeast; Ca, *Candida albicans*, another yeast.

2.4.2 Expression analysis

The spatial expression of the *POLδ1* gene was assessed by semi-quantitative RT-PCR and in plant lines that express the *GUS* reporter gene driven by the promoter region of *POLδ1* spanning the non-coding sequences between the two genes at5g63950 and at5g63960 (see Figure 22, page 74). Two splice-variants of the *POLδ1* transcript were found (see page 73); their tissue-specific expressions were analysed by RT-PCR using common and specific primers for either transcript. Expression of *POLδ1* was detected in all assessed tissues: roots, rosette leaves, stems, flowers and callus culture (Figure 29A). Higher steady-state levels of transcripts of both splice forms were assigned to tissues with actively dividing cells such as flowers and callus cultures. Although the applied method did not allow a quantitative assessment, the transcript levels for the spliced form were found to be elevated in all analysed tissues. This mRNA may

therefore code for the more important product of the *POLδ1* gene, presumably with the biological function of a replicative DNA polymerase.

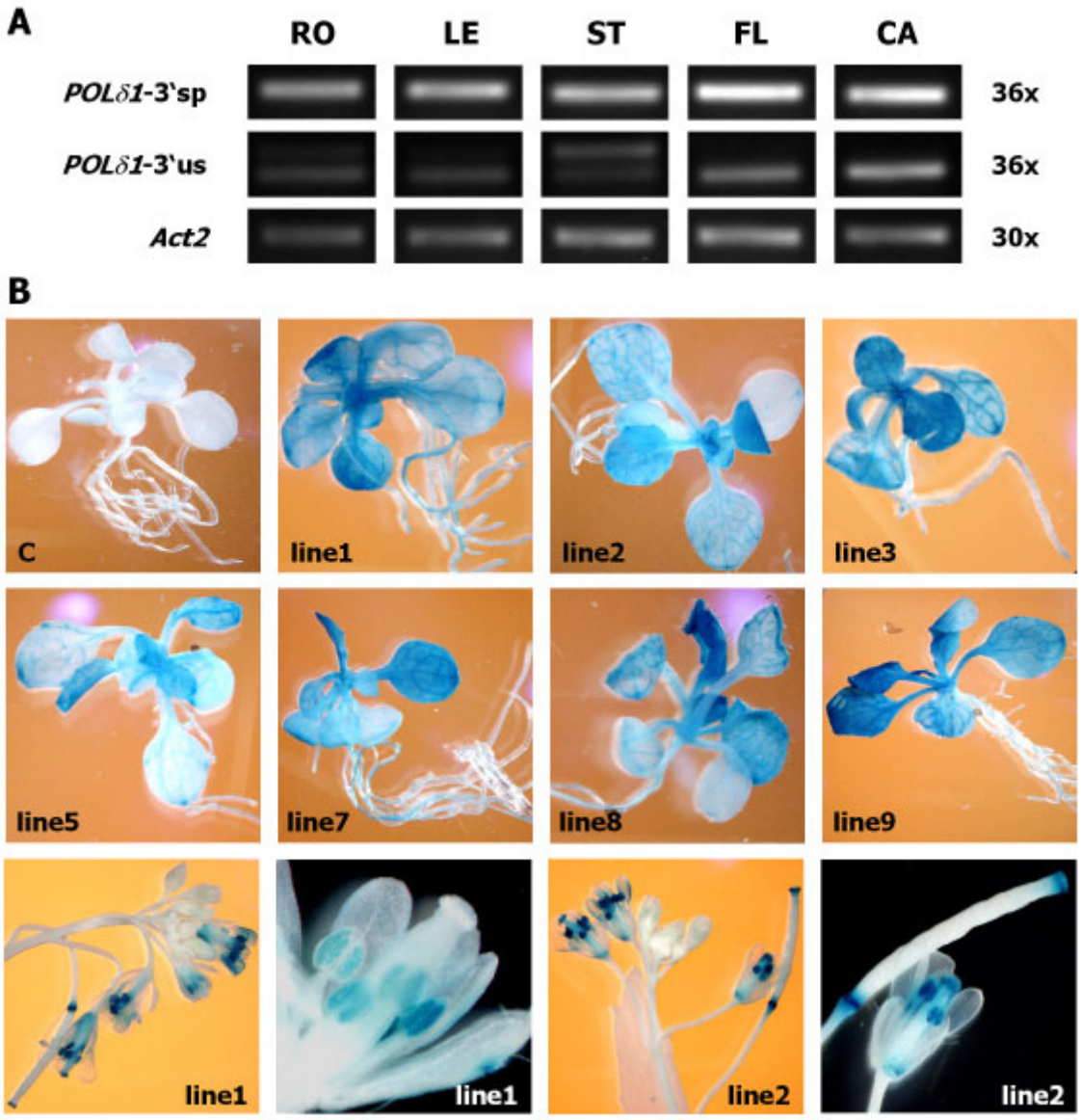


Figure 29: The expression analysis for the *POLδ1* gene
A. Semi-quantitative RT-PCR was used to amplify the spliced (*POLδ1*-3'sp) and the variant (*POLδ1*-3'us) transcript of *POLδ1*. Steady-state levels of *POLδ1* transcripts were analysed in total RNA isolated from roots (RO), rosette leaves (LE), stems (ST), flowers (FL) and callus culture (CA). The constitutive expression of the *Act2* gene was used as control (last row). The last column indicates the number of PCR cycles. **B.** Histochemical analysis of GUS activity in transgenic lines carrying a *POLδ1* promoter-*GUS* fusion construct. Wt plants were used as negative control (C). Plants were analysed in developmental stages of 4-6 leaves (1st and 2nd row) and flowering (last row).

The expression data derived from RT-PCR were confirmed by the analysis of transgenic lines containing the *POLδ1* promoter-*GUS* fusion. Most of the assessed lines revealed similar GUS expression patterns (Figure 29B). GUS activity was detected in virtually all

tissues. The highest activity could be assigned to the meristematic region and to emerging leaves, whereas in cotyledons the expression level was found to be significantly lower. An interesting *GUS* expression pattern was observed in two out of five assessed lines: mature pollen grains, the stigma and also future breakage zone of the growing silique revealed high GUS activities. Although this expression pattern was only seen in two lines, it correlated well with the increased *POLδ1* expression of flowers, which was measured by RT-PCR (Figure 29A).

2.4.3 The analysis of *POLδ1* knock-down plants

In addition to the HR analysis of allelic mutants and complemented lines, several transgenic Arabidopsis lines in a HR reporter background were generated, in which the expression of *POLδ1* was down-regulated. The reduction of the RNA steady-state was intended to be achieved by the expression of inversely repeated *POLδ1* sequences producing an intron-spliced hairpin transcripts. Those were shown to lead to an efficient post-transcriptional degradation of the aberrant double-stranded and also of the endogenous RNA, a process termed RNA interference (RNAi) or post-transcriptional gene silencing [Smith *et al.*, 2000; Stoutjesdijk *et al.*, 2002]. Considering that *POLδ1* is an essential gene, two different types of vectors were used to express the RNAi-promoting transcript: the rather weak constitutive *A.tumefaciens Mannopine synthase* 2' promoter (c-RNAi lines) [Hajdukiewicz *et al.*, 1994; cloning described in: Fritsch, 2004] and the alcohol-inducible expression system (i-RNAi lines) [Caddick *et al.*, 1998; Roslan *et al.*, 2001; Maizel and Weigel, 2004].

Only a few T1 transformants for the constitutive *POLδ1*-RNAi construct were obtained; many of them exhibited a severe growth inhibition and developmental abnormalities, which often resulted in the production of few or no seeds (Figure 32A and data not shown). Lines that produced sufficient seeds were analysed for their HR frequency in the T2 generation. All families revealed significantly more HR events per plant than plants of control lines (Figure 30A). The increase of HR of the individual c-RNAi families ranged from about 7 to more than 40 fold (Table 10). Although the induction of the *POLδ1* down-regulation by the i-RNAi construct upon adding ethanol could not be detected (data not shown), eight out of ten analysed T2 families exhibited a strong increase of HR even without induction. The leakiness of the transactivation system may have been sufficient to reduce the *POLδ1* expression, yielding the observed enhancement of HR levels. Indeed, a comparable decrease of the steady-state level of *POLδ1* transcripts was observed for the c-RNAi as well as for the not

induced i-RNAi lines (see Figure 47, page 134). Compared to the c-RNAi lines, the HR increase of most i-RNAi lines was found to be less pronounced: it was about 2 to 20 times higher (Figure 30B and Table 10). Lines 2 and 10 showed about 50 times more HR events than control plants, which can be compared to the HR induction of the c-RNAi lines. In contrast to them, no phenotypic alterations were observed in these two lines, which indicated that a strong increase in HR frequency did not necessarily correlate with growth inhibition.

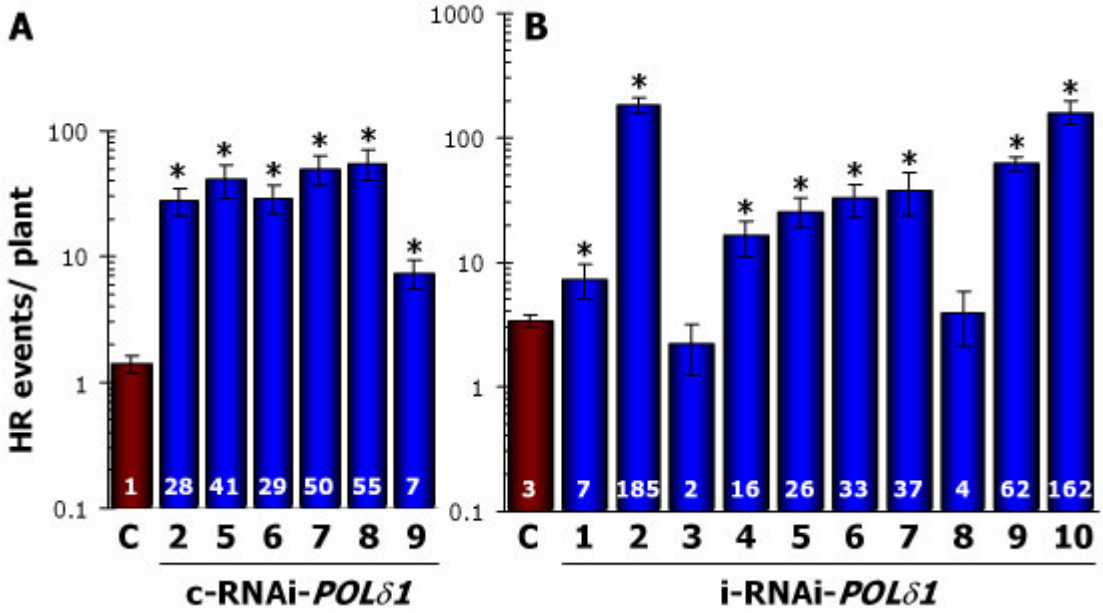


Figure 30: The HR frequencies of Arabidopsis RNAi-*POLδ1* plants
The number of intra-molecular HR events per plant was assessed in control lines (brown) and in lines expressing the *POLδ1* RNAi-promoting hairpin transcript driven by a constitutive (c-RNAi) (**A**) or an inducible (i-RNAi) (**B**) promoter. Asterisks mark statistically highly significant increased HR.

The enhancement of HR frequencies in some of the *POLδ1*-RNAi lines was found to be not ubiquitously strong in different plant tissues. A more pronounced increase of HR level in the cotyledons resulted in the strong hyper-recombination phenotype of most RNAi lines (Figure 31A, B). In wt plants, intra-molecular HR events found in cotyledons contributed predominantly to the total number of HR events of a plant when assessed at the developmental stage of about 4-6 true leaves. In fully developed rosettes more HR events were observed in true leaves than on cotyledons; they principally contributed to the HR frequency of the whole plant. The relative contribution of HR events in these tissues to the overall increased HR phenotype varied in the individual *POLδ1*-RNAi lines, which were accordingly classified into different phenotypic categories. 1: lines with a HR enhancement solely in cotyledons; 2: lines with

enhanced HR in true leaves; 3: lines with a stronger increase of HR in cotyledons than in true leaves, the latter of which becomes evident only later in development; 4: lines with comparable HR increase in cotyledons and true leaves independent of the developmental stage (Table 10).

RNAi line	HR increase rosette stage	Growth inhibition	Cotyledon HR phenotype	class
line 1445, control	-	-	-	-
c-RNAi 1	na	++, few seeds	na	na
c-RNAi 2	20.3	+++	++	na
c-RNAi 5	29.2	++	+++	3
c-RNAi 6	21	++	+++	1
c-RNAi 7	35.7	+/-	+++	3
c-RNAi 8	39.3	+/-	+++	4
c-RNAi 9	5.3	-	-	2
i-RNAi 1	2.1	-	+	1
i-RNAi 2	54.1	-	+++	4
i-RNAi 4	4.8	-	++	3
i-RNAi 5	7.5	-	++	3
i-RNAi 6	9.6	-	++	3
i-RNAi 7	10.9	-	++	4
i-RNAi 9	18.2	-	+++	4
i-RNAi 10	47.4	-	+++	4

Table 10: Summary of phenotypes of *POLδ1*-RNAi lines
Growth inhibition, HR phenotypes of cotyledons and the induction of HR was assessed for the individual lines and they were classified according to their spatial and temporal HR phenotype.

The tissue-specific enhancement of HR in line i-RNAi 1 represented a class 1 plant. This line showed a strong hyper-recombination phenotype in cotyledons but no induction of HR in true leaves even at later developmental stages (Figure 31A, B). The effect of *POLδ1* down-regulation appeared to be restricted to cotyledons; this phenotype was observed for lines bearing the constitutive or the inducible RNAi construct and independently of their influence on plant growth (Table 10). Only one line of class 2 was isolated, lacking the strong phenotype in the cotyledons but showing a moderate 2.1 times HR increase of the whole plant. This was comparable to the HR phenotypes of the various *polδ1* KO alleles. The majority of the *POLδ1*-RNAi lines belonged to the classes 3 and 4, revealing a hyper-recombination phenotype in the cotyledons and also a more or less pronounced HR increase in true leaves, which became evident at different stages during the development (Table 10). The i-RNAi lines 5 and 6 showed the class 3-typical HR enhancement only in the cotyledons of young plants, whereas at a later time-point the increases were also observed in true leaves, preferentially in older leaves (Figure 31A, B). In contrast, the line i-RNAi 10 categorised into class 4 exhibited a similarly increased HR frequency in cotyledons and in true leaves, already

observed shortly after they had emerged. Notably, this strong HR increase was never seen in small and unexpanded leaves and its establishment generally started on the older leaves.

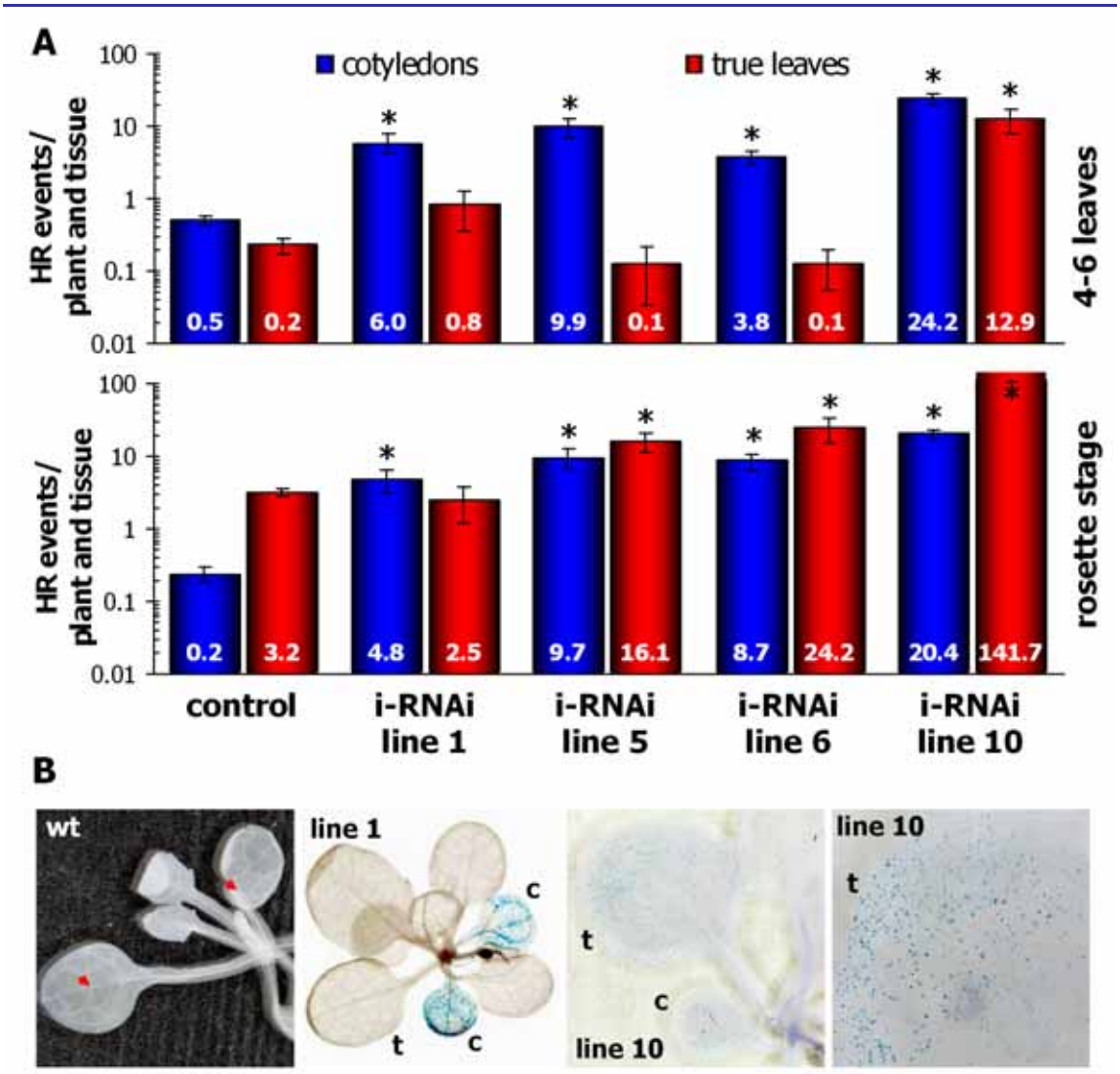


Figure 31: The tissue-specific increase of HR in *POLδ1*-RNAi lines
A. Establishment of the increased HR frequency of exemplary lines are shown for an early and a late developmental stage in the top and the bottom panel, respectively. Error bars indicate standard errors of HR events per plant of each line. Asterisks mark significant differences to control plants. **B.** Representative picture of visualised HR events in wild type and *POLδ1*-RNAi lines. c, cotyledons; t, true leave.

Differentiation between class 3 and 4 RNAi lines was difficult in some cases since the numbers and the temporal appearance of the HR events were found to vary significantly between individual plants and experiments. Therefore, a precise temporal assessment of the phenotypic constitution could not be done, leading to a quite arbitrary classification that reflects the observed HR frequencies at two time-points. Interestingly enough, the strength of HR induction in the whole plant seemed to

correlate with the temporal appearance of HR events in true leaves: most of the class 4 lines revealed strong and early increases of HR in both cotyledons and true leaves (Table 10 and Figure 30). The temporal establishment of HR inductions may reflect the strength of the respective knock-down alleles.

Dwarf phenotypes were observed for some of the constitutive *POLδ1*-RNAi lines but not for the "inducible" ones (Figure 32A and data not shown). The c-RNAi lines 2, 5 and 6 revealed the strongest growth inhibition: plants of these families were significantly smaller than wt plants at all developmental stages. Strikingly, the number of rosette leaves and the time of flowering appeared to be less affected. Leaves emerged more or less in synchrony with those of control plants but failed to grow further and to expand. In addition, the leaves - mainly older ones - had often a pale appearance. Ambivalent phenotypes were observed among the plants of the c-RNAi line 1: a portion of them phenotypically resembled mutant plants of lines 2, 5 and 6, for others the size reduction was less prominent and their leaves even appeared greener, rounder and thicker than those of wt plants. Considering the crucial role of the DNA polymerase δ in genome replication, the failure of DNA replication was hypothesised to cause growth reduction of *POLδ1*-RNAi plants. Plant replication mutants such as *bru1* [Takeda *et al.*, 2004], *fas1*, *fas2* and *msi1* that are mutated in the genes coding for the three subunits of the chromatin assembly factor 1 (CAF1) [Kaya *et al.*, 2001; Hennig *et al.*, 2003] or the partially complemented homozygous *polδ1-1* mutants (see page 79) exhibited typically reduced growth and misshaped organs such as fasciated stems. These phenotypes may stem from epigenetic changes, unequal cell division or meristematic cell death triggered by reduced or failed replication.

However, the dwarf plants of the *POLδ1*-RNAi lines such as c-RNAi 1, 2, 5 and 6 did not resemble the phenotypes of replication mutants described above and revealed normal morphology, suggesting a distinct causal effect independent of the meristematic DNA replication. Plants have evolved a special way to increase the metabolic capacity of their cells by endoreduplication that comprises genome amplification without subsequent cytokinesis. A higher ploidy level of cells correlates with the cell and even with plant size [Sugimoto-Shirasu and Roberts, 2003]. The down-regulation of *POLδ1* expression may have interfered with endoreduplication and thereby prevented cellular expansion. This could reduce the energy assimilation of these plants, which would explain the dwarfisms and also the pale leaves. In order to

test this hypothesis the nuclear DNA content of cells isolated from wt and *polδ1* alleles was analysed by fluorescence activated cell sorting (FACS).

DNA of cells from various tissues and of different age was labelled with propidium iodide and the nuclear DNA content was measured by FACS analysis. The strongest effect of knocking-down *POLδ1* expression on the HR frequency was observed in the cotyledons of most RNAi lines (see above). The DNA content of nuclei isolated from wild type cotyledons showed a distribution with several peaks representing 2C, 4C, 8C, 16C and 32C chromosomal copies (Figure 32B). Significant and reproducible differences in nuclear DNA content of cotyledon cells could not be assigned to any of the RNAi mutant lines, suggesting a minor effect of *POLδ1* down-regulation on the process of endoreduplication (Figure 32B and data not shown). Confirming this notion, the relative distribution of nuclei with a said DNA content did also not differ between control and *POLδ1* mutants in true leaves of various age (Figure 32C and data not shown).

The semi-destructive extraction method and the unbiased staining of any DNA molecule allowed the detection of all DNA containing cellular compartment such as the nucleus, plastids and mitochondria. The plant organelles are distinguishable from the nuclei by lower DNA content and by size, characteristics that can be scored by FACS. In wild type plants, about 40 organelles were counted for each cell represented by the typical fluorescence pattern of a nucleus (Figure 32C). The number of organelles per cell estimated by this method was significantly reduced to 1 to 3 in the c-RNAi lines 2, 5 and 6, which showed the strongest growth phenotypes. Plants of line 1 yielded a divergent outcome: cells of the dark green and slightly smaller plants contained about two times more organelles than wild type cells whereas in plants with more severe growth inhibition the cellular organelle content significantly decreased (Figure 32C, right panel). In contrast, cells of the phenotypically normal c-RNAi line 9 had organelle numbers comparable to wt cells. In conclusion, the estimated organelle content corresponded to the growth and pigmentation phenotype of the different c-RNAi lines, suggesting a causal effect of down-regulation of *POLδ1* expression on organelle replication or maintenance.

Figure 32: The growth reduction and the DNA content of *POLδ1*-RNAi lines
A. Comparison of *in vitro* grown plants with constitutively down-regulated *POLδ1* expression. **B.** Nuclear DNA content of cells isolated from the cotyledons and analysed by FACS. **C.** Estimation of the number of cellular organelles by their DNA contents in control and c-RNAi lines (left panel). The number of mitochondrial and plastid organelles per cell (nucleus) was compared between different lines (right panel). Asterisks indicate significant differences. (Figure on next page)

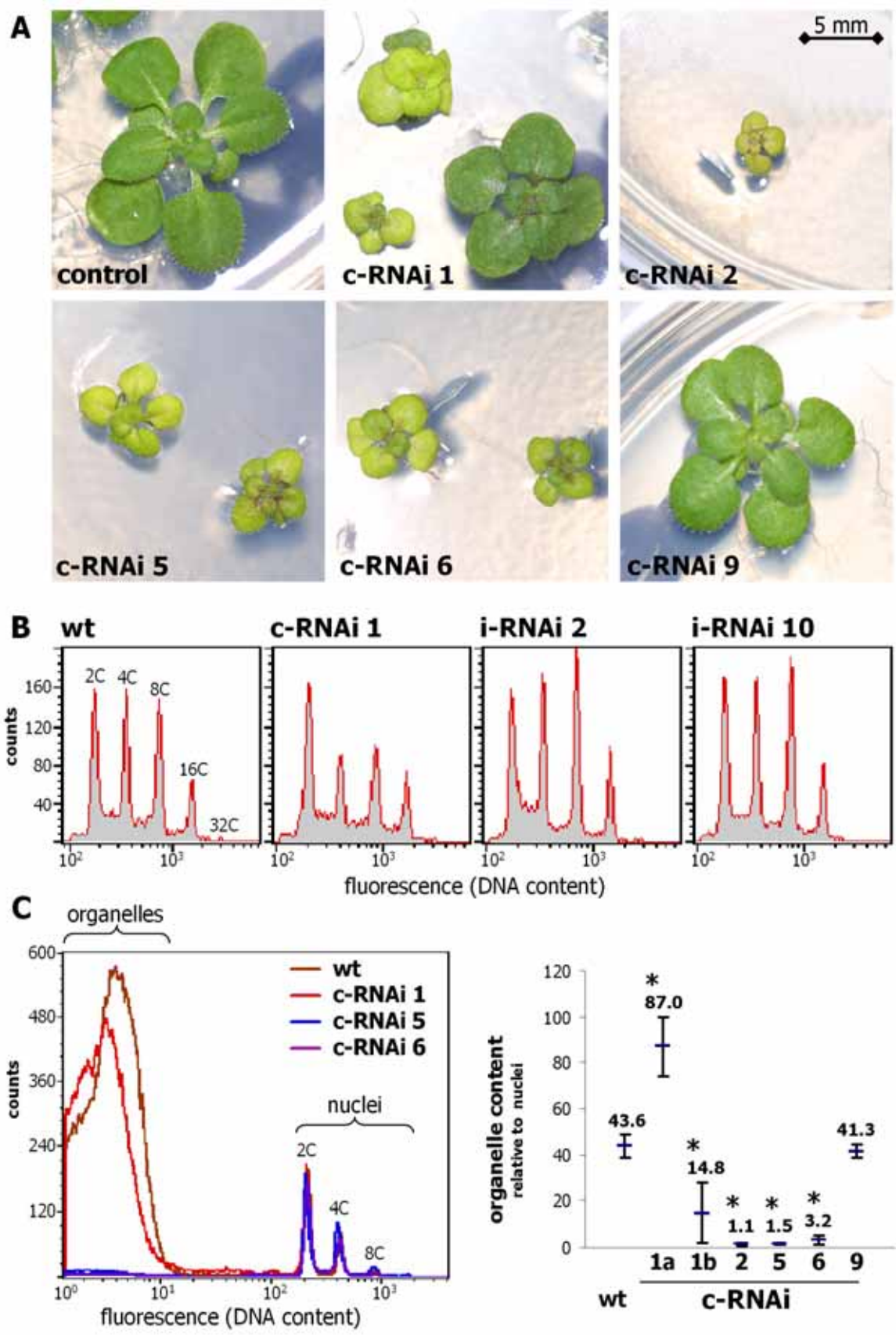


Figure 32: The growth reduction and the DNA content of *POLδ1-RNAi* lines

2.5 The DNA synthesis-recombination connection

The DNA polymerase δ holoenzyme is thought to have two main cellular functions: the replication of the genome and repair synthesis (see Chapters 1.4 and 1.6, pages 10 and 40). Depletion or a reduced level of its largest subunit - POLδ1 - could give rise to genome instability and may therefore result in the increased HR frequency. This was indeed observed for several Arabidopsis *polδ1* mutant alleles. The inhibition of S-phase DNA synthesis would lead directly to abortive replication and growth arrest whereas repair insufficiency would rather result in a prolongation of all cell phases in order to repair remaining lesions. The Polδ1 availability and an increased level of unrepaired DNA damages can be mimicked by treating wt plants with chemical compounds which inhibit DNA synthesis and cell cycle progression or which induce DNA lesions. In order to understand the participation of replication or repair problems in the HR phenotype of the *polδ1* mutants, the influences of various treatments on the HR frequency of wt and of mutant plants were studied.

2.5.1 Inhibition of DNA synthesis and HR frequency

The chemical substance hydroxy-urea (HU) is a potent inhibitor of DNA metabolism depleting the dNTP pool; it is widely used to synchronise cells in the S-phase [Planchais *et al.*, 2000]. It inhibits the ribonucleotide reductase (RNR) which was found to be a key enzyme in the synthesis dNTPs and to be involved in DNA damage response [Tanaka *et al.*, 2000; Chabes *et al.*, 2003]. Lowering dNTPs pools may resemble the effect of lower POLδ1 availability in the *polδ1* mutants and interfere with DNA replication progression which results in arrested replication forks. Arabidopsis plants of the substrate lines 1445 and IC-9C bearing intra- or inter-molecular recombination substrates, respectively, were grown in media with different concentrations of HU for one week. The effect of HU on plant growth and HR frequency was assessed (Figure 33). Growth effects were evident at concentrations higher than 750 μM, which progressively reduced the growth rate of plants with increasing HU concentrations from 1 mM to 10 mM. Concentrations higher than 12.5 mM almost completely blocked cell division and biomass production.

A significant increase of the intra-molecular HR frequency was observed only at HU concentrations that severely affected growth (see Figure 33), indicating a correlation between replication defects and the appearance of recombination events. The most pronounced increase of HRF was found at a HU concentration of about 10 mM, at which the growth of plants was inhibited by 80% compared to that of

untreated ones. At higher HU concentrations the observed HR events dropped to a level below the one of untreated plants. Presumably, this was due to a deleterious effect of low dNTP pools on cell metabolism and division, triggering cell death. However, the effect of HU on the frequency of inter-molecular HR was much less prominent; a doubled HRF was only observed for concentrations ranging from 3 to 50 mM HU. This difference between intra- and inter-molecular recombination suggests the preferential use of repeated sequences within the same DNA molecule to overcome the block of DNA synthesis by HR.

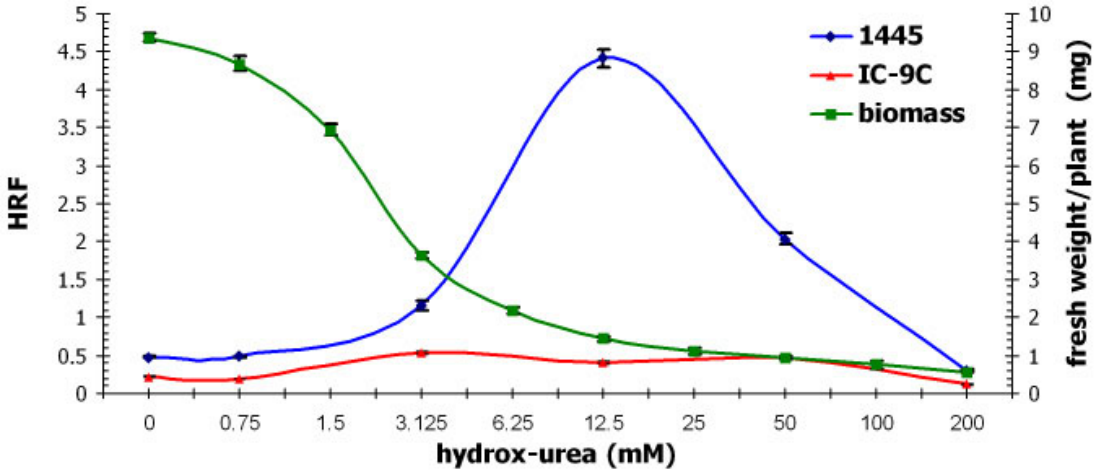


Figure 33: The effect of hydroxy-urea on plant growth and HR
Arabidopsis plantlets were analysed for their reaction on chemical inhibition of DNA synthesis by hydroxy-urea (HU). Fresh weight of plants and the induction of HR were measured in lines bearing the intra-molecular (1445) or the intermolecular (IC-9C) recombination substrates. HRF: homologous recombination frequency (HR events/plants). Error bars indicate standard errors.

The previous experiment showed a significantly increased HR frequency upon depletion of DNA synthesis by HU, preferentially for intra-molecular recombination events (see above). In order to exclude line-specific differences in HR induction, the experiment was expanded to two independent intra- and inter-molecular substrate lines and to other chemicals, which interfere with cell cycle progression or which induce DNA lesions. The Arabidopsis reporter lines 1415 and 1445 monitor intra-molecular HR events [Gherbi *et al.*, 2001] whereas the lines IC-6C and IC-9C specifically measure inter-molecular HR [Molinier *et al.*, 2004b]. In the applied experimental condition, comparable basal levels of HR were observed in both intra- and in both intermolecular HR lines: about 0.9 and 0.5 HR events per plant, respectively (data not shown). This difference may reflect a more frequent use of sequences within the same DNA

molecule to repair spontaneous DSBs than on the homologous chromosome (see page 54). Accordingly, intra-molecular HR was found to be more enhanced than inter-molecular HR upon chemical induction of DSBs by 10 μM bleomycin (BLM) (Figure 34A). At this BLM concentration, the induction of intra-molecular HR was about three fold more pronounced than inter-molecular HR: 43 and 34 times for intra- and 12 and 13 times for inter-molecular HR.

The DSB-induced increase of HR by BLM treatment was taken as baseline for HR induction in the following treatments with the DNA crosslinking chemicals mitomycin-C (MMC) and cis-platinum (CIS) as well as with the inhibitors of cell cycle progression HU, aphidicolin (APC) and mimosine (MIM) [Planchais *et al.*, 2000]. Experiments with 25 μM CIS and 10 μM MMC served as additional control experiments for the HR induction by DNA damage (Figure 34B). Strongly increased HR frequencies were observed in all lines upon treatment with CIS. Interestingly enough, the increases were more pronounced in the inter-molecular HR substrate lines. Either the threshold level of intra-molecular HR was reached at this CIS concentration or the repair of CIS-induced DNA lesions necessarily involved extra-molecular sequences. Indeed, a non-linear correlation between the HRF and the applied doses was observed for many DNA damaging agents (pers. com.: O. Fritsch and J. Molinier). In contrast to an induction by CIS, the HR levels were found to be similarly enhanced by MMC in all analysed substrate lines, confirming a comparable behaviour if standardised to the DSB-inducing chemical BLM.

APC specifically inhibits Polα and Polδ by binding to them and by interfering with their processivity. Thus, both HU and APC block DNA synthesis but their mode of action is quite divergent. In contrast, MIM blocks the G1/S-phase transition by an unknown mechanism and served as supplementary control in this experiment. These inhibitors of cell cycle progression were applied at concentrations that reduce the plant growth by about 50-70% (see above, data not shown): 6.25 mM HU, 50 μM APC and 10 mM MIM. In agreement with the previous HU experiment, no increase of inter-molecular HR was observed whereas the levels of intra-molecular HR in line 1415 and 1445 were enhanced 10 and 6 times, respectively (data not shown). Blocking S-phase DNA synthesis by HU and comparing the HRF to BLM treatments, the relative induction was about 20% in both intra-molecular substrate lines (Figure 34C). These increases were found to be significantly different to the induction of inter-molecular HR, underlining the preferential use of intra-molecular recombination to resolve stalled replication forks. Comparable results were obtained when the DNA polymerase

inhibitor APC was applied, although line 1445 did not exhibit a significant difference to the inter-molecular lines (Figure 34C). In comparison to HU treatments, the HR enhancement by APC was stronger in all tested reporter lines: about 2 and 4 times for intra- and inter-molecular recombination, respectively (Figure 34C and data not shown). Blocking the cell cycle at the entry to the S-phase by MIM resulted in only minor differences between intra- and inter-molecular HR, although the absolute HRF was found to be slightly increased in all lines compared to untreated plants (Figure 34C and data not shown).

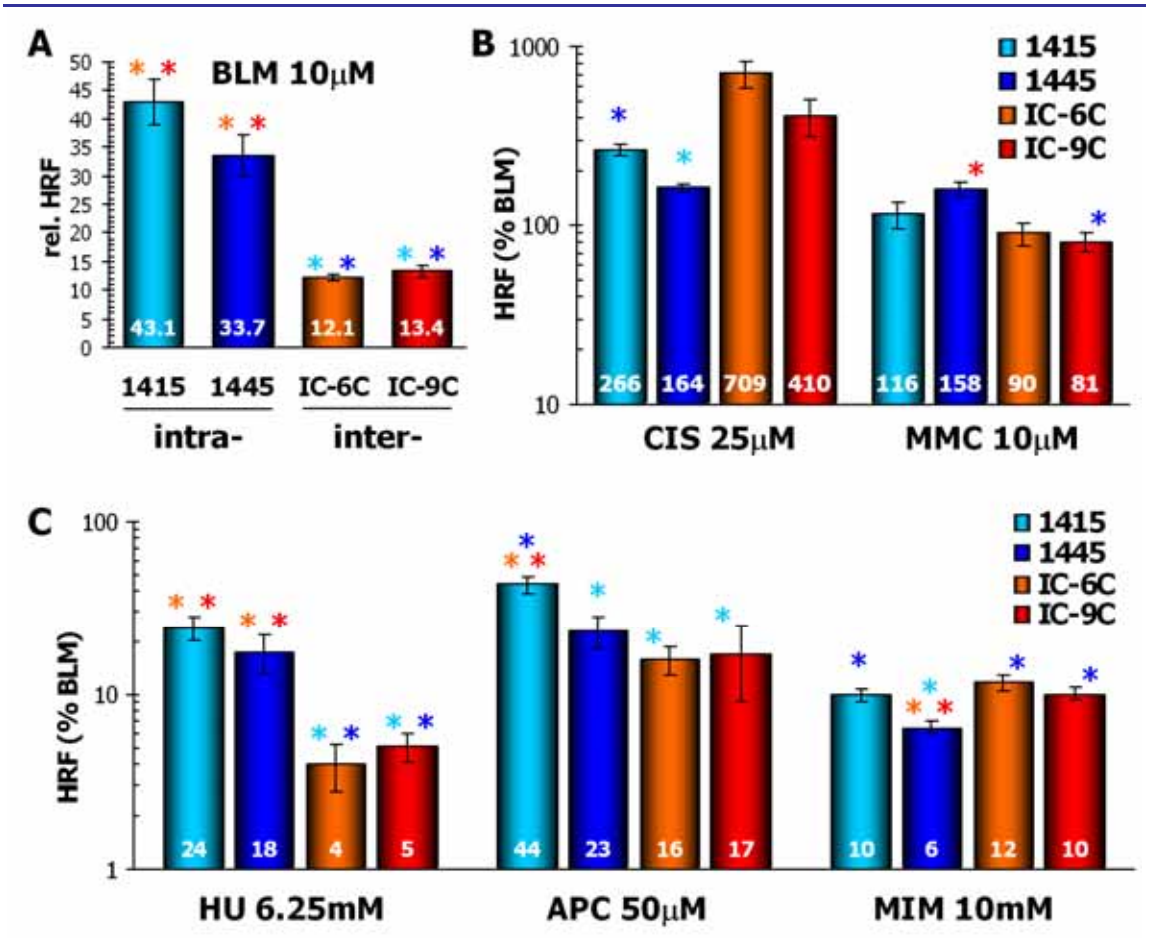


Figure 34: The induction of intra- and inter-molecular HR by chemicals
One week old plantlets bearing intra- (1415, 1445) or inter-molecular (IC-6C, IC-9C) HR substrates were incubated with the respective chemicals for three and further grown for 5 days. **A.** The HR frequencies (HRF) upon induction of DSB by 10 µM bleomycin (BLM) were analysed for all substrate lines and served as an induction baseline for the subsequent experiment. **B.** Control experiments: DNA lesions were induced by the cross-linking agents cis-platinum (CIS) and mitomycin-C (MMC) and the HR induction was standardised to the BLM treatment. **C.** Relative HRF measured in plants treated with the DNA-synthesis blocker hydroxy-urea (HU) and aphidicolin (APC) and by the G1/S-phase transition inhibitor mimosine (MIM). Asterisks mark significant difference to the line of the respective colour and error bars indicate standard errors of 4 independent experiments.

In conclusion it can be said that the prolongation of a cell cycle phase yielded a comparable response in increased intra- and inter-molecular HR. On the other hand, blocking of DNA replication by two different modes resulted in a more pronounced enhancement of intra-molecular recombination. The effect of these treatments on the HR was also assessed in a substrate line with direct repeats of the homologous sequences (1406), which monitor intra- and also inter-molecular HR events (Figure 13, page 60). BLM was found to induce an increase in HR frequency of about 75 times whereas the relative induction to BLM-induced DSB by HU and APC were about 10 and 24%, respectively. These HR inductions corresponded to enhancement levels in-between the observations for intra- and inter-molecular recombination (data not shown).

2.5.2 Inter- and intra-molecular HR behaviour of *polδ1* mutants

The previous experiment suggested the preferential use of homologous sequences located on the same DNA molecule to overcome S-phase blockage. The applied chemical inhibitors result in abolished DNA synthesis and thus in stalled replication. In order to evaluate whether the hyper-recombination phenotype of the *polδ1* mutants may originate from DNA synthesis insufficiencies similar to the ones caused by chemical inhibition, the effect of the *polδ1-1* allele on the HR level was analysed in an intra-molecular (1445) and in an inter-molecular (IC-9C) substrate line (see page 60). Furthermore, the number of HR events per plant was analysed at different plant ages: from 2 true leaves to the stage with fully developed rosettes.

In this experimental setup, wild type plants revealed an increase of HR events per plant over time (Figure 35). Assuming 10-12 leaves at rosette stage in these growth conditions, about 0.1 and 0.06 HR events per leaf were counted for lines 1445 and IC-9C, respectively. This suggested that the HR frequency of both lines correlated with the leaf and presumably total cell number of a plant, which means a constant recombination frequency per cell over time. At the two leaf stage *hw17* mutant plants showed 0.3 intra-molecular HR events per plant which was only slightly higher than in control plants. Subsequently, *hw17* mutants accumulated more HR events than control plants (Figure 35, left panel); the most pronounced difference (over 7 times more) was observed in plants at the 6 leaf stage. Furthermore, these findings showed that the *polδ1-1* mutation also caused an increased HRF in Arabidopsis line with an independent substrate locus. This excluded the possibility that a direct interaction of the 50B substrate and the *hw17* locus resulted in the hyper-recombination phenotype. In

contrast to an increased intra-molecular HR, the same mutant allele did not yield any significant alterations in HR frequency in the inter-molecular substrate line (Figure 35, right panel). A slight and not significant increase could only be detected at later stages of development whereas earlier the level of HR resembled the one of wt plants.

The result of this experiment showed that the mutation in *POLδ1* in *hw17* plants is leading to elevated frequencies of recombination between homologous sequences of the same DNA molecule. Interestingly enough, chemical blocking of DNA synthesis of which POLδ1 is an essential player also yielded a similar specificity for intra-molecular HR. This suggests a correlation between DNA synthesis or replication inhibition and the appearance of the hyper-recombination phenotype in *polδ1* mutant background.

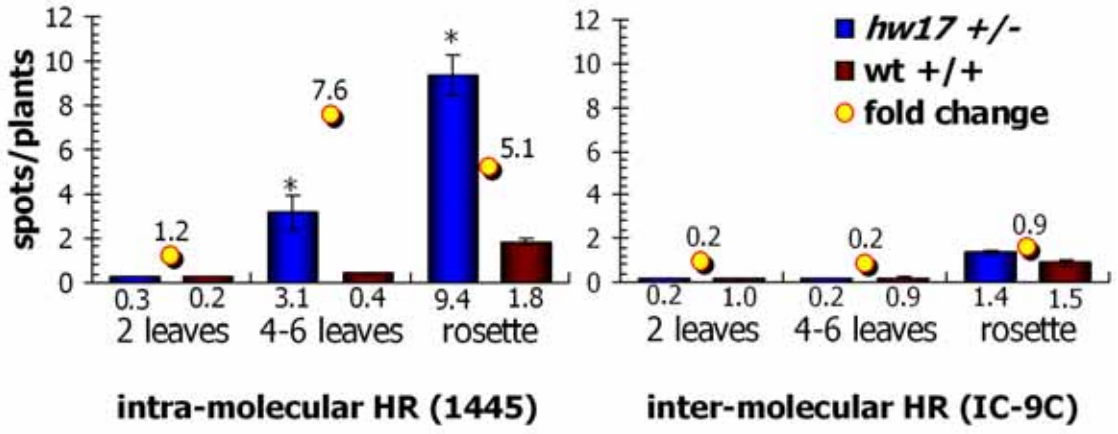


Figure 35: The intra- and inter-molecular recombination frequencies in *hw17*

The *hw17* mutation was introduced into line 1445 (left panel) and line IC-9C (right panel) which contain a *GUS*-based substrate for intra- and inter-molecular recombination, respectively. The number of HR events was assessed at different stage of development and compared to the one of segregating wt families. Error bars indicate standard errors. Asterisks indicate statistically sound differences.

Specificities in the enhancement of HR as found in the distinct Arabidopsis substrate lines may not only reflect the differential use of template sequences but also repair according to the different models (Chapters 1.4.1.2 and 1.8.3, pages 14 and 58). The relative orientation of the repeated sequences to each other could influence the creation of a molecular event that results in the restoration of a functional marker gene. In order to elucidate the molecular mechanism of the HR induction in *polδ1* plants in more detail, the effect of dominant *POLδ1* mutations (*polδ1-1, polδ1-3*) on the HRF in various hemizygous Arabidopsis reporter lines were tested. As expected for a hemizygous recombination substrate in the offspring, the number of detected HR events dropped in all reporter lines crossed with wt plants in comparison to selfed ones

(Figure 36). In the reporter line 1415 (intra-molecular HR, indirect repeats, like 1445 and 50B), about 50% less recombination events were observed, confirming that they happened solely intra-molecularly. The substrate in line 1406 allows the detection of inter- and also of intra-molecular events that follow the repair models DSBR or SSA. Line IC-9C monitors recombination between sister chromatids or homologous chromosomes [Molinier *et al.*, 2004b]. The HR frequency of hemizygous and homozygous reporter plants for both substrates lines did not change significantly.

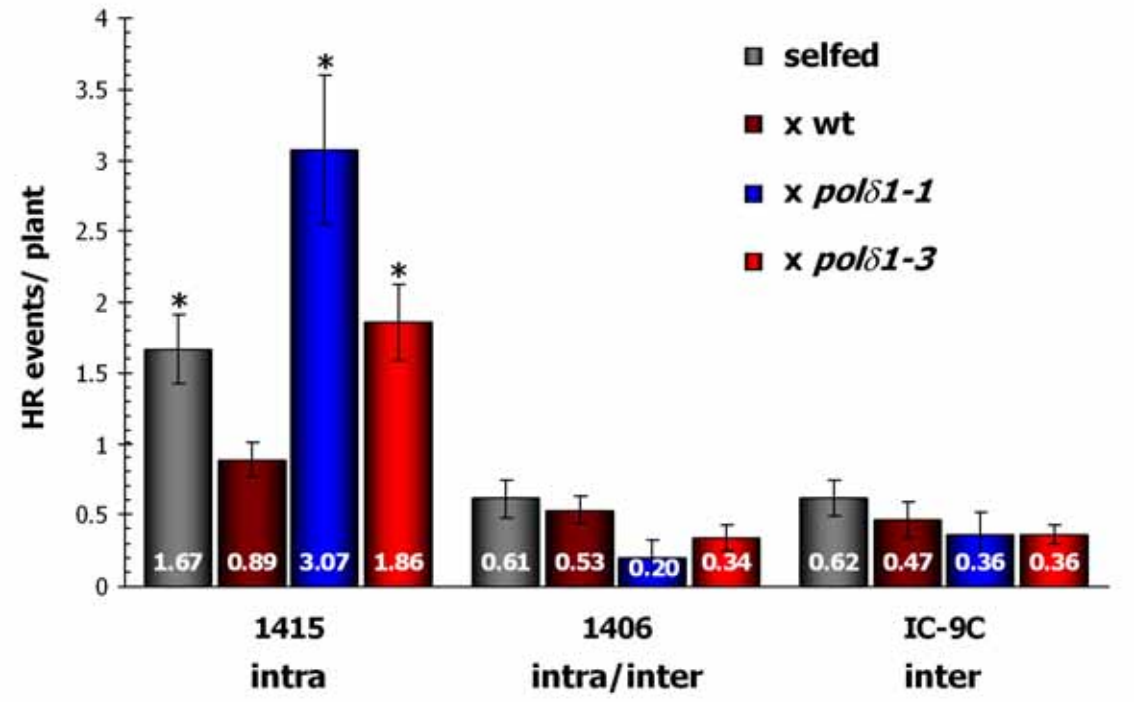


Figure 36: The influence of mutations in *POLδ1* on hemizygous HR substrates
Two dominant *polδ1* alleles were crossed into three substrate lines with different spatial arrangement of the homologous template sequences which detect different types of recombination events. The influence of the *POLδ1* mutations on the HR frequencies was assessed in the F1 generation. Asterisks indicate statistically sound differences to HR substrate plants derived from crosses with wt plants.

Confirming the results of the previous experiments, both tested *polδ1* mutant alleles yielded a significant increase of intra-molecular HR whereas no alteration was found in hemizygous inter-molecular substrate lines (Figure 36). Comparable results were also obtained for crosses of the two *polδ1* alleles with independent substrate lines (data not shown). Intriguingly, both dominant *polδ1* mutant alleles exhibited no or rather a decrease in HR frequency in the substrate line 1406, which is able to detect intra-molecular events like 1415. This data indicates that the relative orientation of the homologous template sequence and not its presence on the same or on a different

DNA molecule defines the substrate line-specific enhancement of HR caused by mutations in *POLδ1*. Unfortunately, this finding could not yet be reproduced in an independent reporter line since a suitable one was not available. Although a high HRF was observed for line 1406 upon DSB induction by BLM, particularities of this line cannot be excluded (data not shown).

2.5.3 Induction of HR in a *polδ1* mutant background

Challenging plants with chemicals or radiations that cause DNA damage was shown to result in elevated frequencies of HR [Puchta *et al.*, 1995b; Ries *et al.*, 2000a; Molinier *et al.*, 2004b]. Such genotoxic treatments induce either DSBs or produce other types of DNA damage which could be converted to substrates for repair by HR. If not repaired these DNA lesions may potentially provoke strand breaks and replication arrest in the S-phase, resulting in substrates for homology-based repair. Those mechanisms may explain the increased HR frequency of mutant plants impaired in the NER repair pathway [Molinier *et al.*, 2004a]. Presumably, alternative repair mechanisms are engaged to remove DNA damages that remained in the genome for a prolonged time. Also, the induction of HR frequency upon genotoxic challenge was found to behave differently in a repair gene mutants and wild type plants [Molinier *et al.*, 2004c]. Apart from its role in DNA replication, POLδ1 was anticipated to function in most repair pathways (Chapter 1.4, page 10). It could be argued that inefficient repair in the G1-phase results in the accumulation of DNA lesions, which leads to the frequent stalling of replication in the S-phase and thereby to the HR phenotype of *polδ1* mutants. The induction of the HR frequency in *polδ1* mutants upon genotoxic treatments could thus give some insights into the impact of POLδ on DNA repair.

Irradiation of plants with UV-C induces mainly CPDs and 6-4PP, which are mostly repaired by photolyases and by the NER pathway. Wild type (50B) and *hw17* plants were irradiated with 5 kergs of UV-C and the number of intra-molecular HR events was analysed and compared to untreated plants of both lines. The HR frequency of *hw17* mutant plants did not react at all to UV-C exposure and revealed about 4.5 events per plant whereas control plants doubled the number of HR events (Figure 37A). Since the applied dose of UV-C irradiation severely inhibited plant growth (Figure 37B), the induction of the HR frequency was certainly underestimated by the calculation of the number of events per plant. However, the data clearly indicated that repair by HR in *hw17* mutants was less inducible by UV-C irradiation than in control plants.

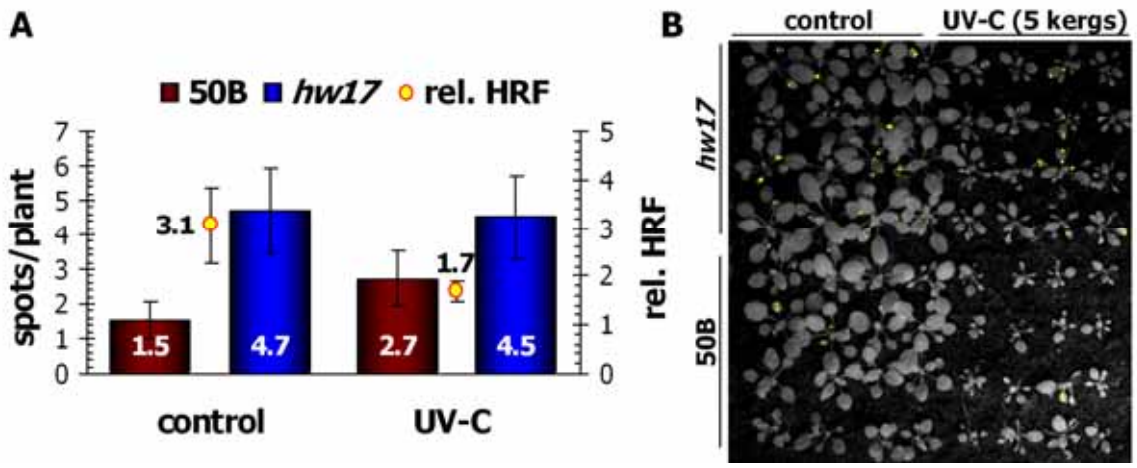


Figure 37: The HR induction by UV-C irradiation
A. The number of HR events in wt and in *hw17* mutant plants was compared between UV-C irradiated and untreated control populations. **B.** Representative picture of the UV-C irradiation experiment, which shows the experimental setup and HR events of treated and untreated lines.

Chemical inducers of DNA adducts and breaks as well as DNA synthesis inhibitor were used to further study the HR response of *polδ1* mutants. In this experiment, the behaviour of intra-molecular recombination in the substrate line 1445 was analysed in the dominant *hw17*, in the recessive *polδ1-2* and in an RNAi-*POLδ1* alleles. Plants of the c-RNAi-*POLδ1* family 9 were chosen since they did not show the strong hyper-recombination phenotype in cotyledons and no growth inhibition. They exhibited a moderate increase of HR similar to the two T-DNA *POLδ1* mutants (see Chapter 2.4.3, page 85). In fact, without treatment this line did not show any change in HR frequency compared to the 1445 control in this experiment assessing two weeks old seedlings (Figure 38A, left panel). In contrast, the T-DNA KO-alleles *hw17* and *polδ1-2* revealed increased levels of HR by a factor of 5 and 3, respectively. All lines were tested for their capacity to react with increased HR frequency to DNA DSBs induced by bleomycin. Compared to untreated wt plants, an induction of the HR frequency was found in all lines, ranging from 14 to 24 times more events (Figure 38A, right panel). Taking the elevated level of spontaneous HR events of the different *polδ1* alleles into consideration, the induction of HR by BLM in all of them was weaker than the ones of control plants (Figure 38B). Thus, the induction of HR by bleomycin treatment seemed to be reciprocal to the basal HR frequencies of the untreated plants. This may indicate that the capacity of *polδ1* mutants to repair DSBs by HR reached a level which cannot be exceeded even by the induction of additional breaks. Alternatively, DSB repair by

HR may be reduced in the mutants due to the lack of sufficient POLδ1 for repair synthesis.

A similar tendency was observed for the treatments with low levels of cis-platinum (CIS) and methyl methanesulfonate (MMS). The HR frequencies in the *polδ1* mutants were less inducible than in control plants. The *hw17* mutant even did not react at all to CIS at the applied concentration of 1 μM. Interestingly enough, these differences were found to be less pronounced in MMS treated plants. This chemical substance mainly produces oxidative DNA modification that can also be repaired by DNA polymerase δ-independent pathways.

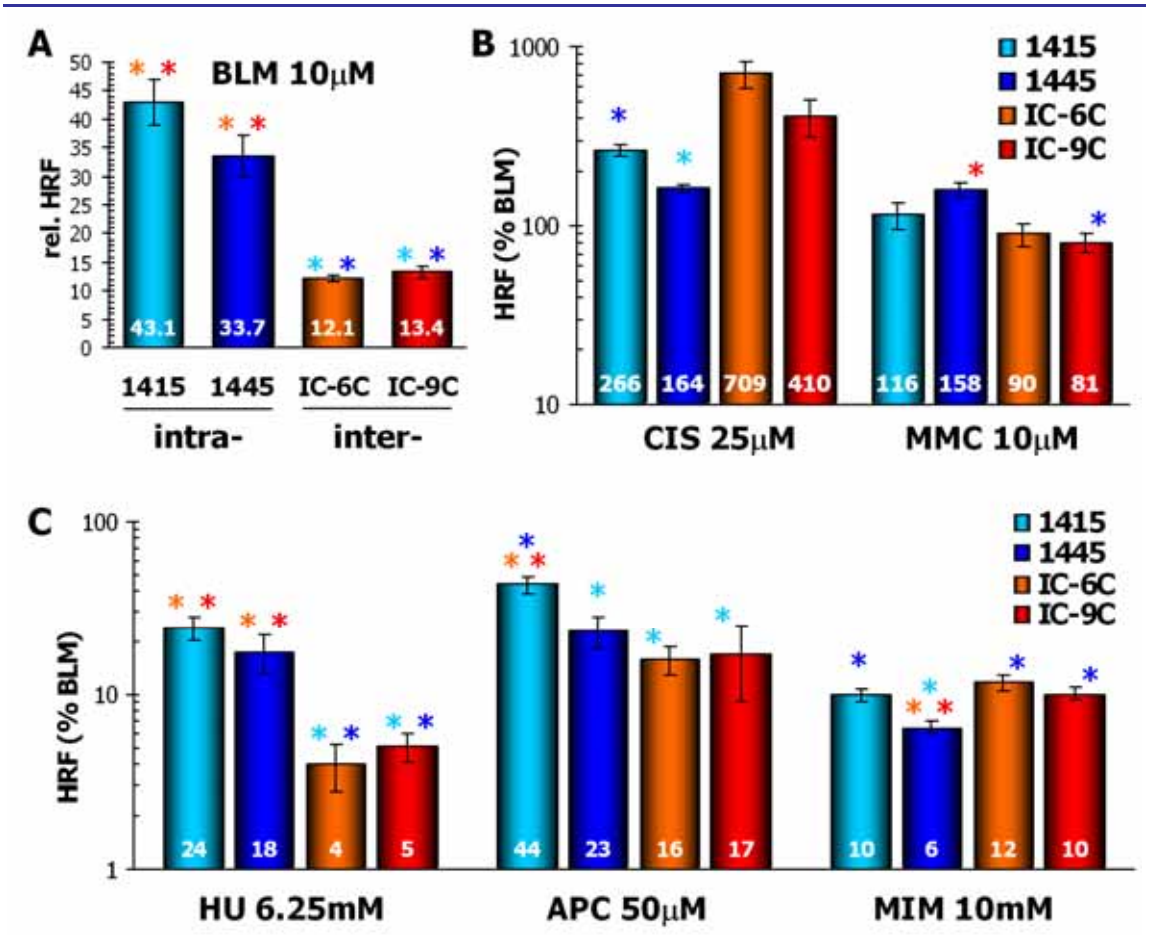


Figure 38: The HR induction by chemical challenge in *polδ1* mutant alleles
The HR behaviour of three *polδ1* alleles, two T-DNA and an RNAi mutant, upon treatment with the DNA synthesis blocker hydroxy-urea (HU), the DSB inducer bleomycin (BLM), the DNA crosslinker cis-platinum (CIS) and the alkylating agent methyl-methansulfonate (MMS) was assessed. **A.** HR frequencies of mutant lines compared to unchallenged control lines (line 1445). BLM as potent inducer of DSBs and thus HR was used to standardise the reaction of the different lines. **B.** The induction of HR frequency by several treatments standardised against untreated plants of the respective lines. Error bars indicate the standard errors of four independent experiments. Asterisks mark statistically significant differences.

In wt plants, the inhibition of DNA synthesis by 1 mM hydroxy-urea showed an increased frequency of HR of about 5 times (Figure 38B). The HR induction in the two T-DNA KO alleles - *hw17* and *polδ1-2* - reacted to the same extent which excluded an additive effect of transcriptional down-regulation of *POLδ1* and further replication inhibition. In contrast to these two alleles, the HR frequency in the c-RNAi 9 line was induced 5 times stronger than in wt plants. One may speculate that the pool of POLδ1 protein in this line was reduced more than in the others, therefore resulting in the observed differential behaviour of HR induction upon treatment with hydroxy-urea.

These HR induction experiments with genotoxic treatments and with a DNA synthesis inhibitor revealed a generally diminished response of *polδ1* mutants compared to control plants. This may indirectly indicate that POLδ1 participates in DNA repair by HR, which is partially impaired in the *polδ1*. However, due to the enormous variation between individual plants and also between experiments many of the obtained data cannot be considered statistically significant. Additional dose-dependent induction experiments should be performed in order to substantiate the data.

Chapter 3 Discussion

There is not nearly as much information available about the DNA metabolism of plants than there is about yeast or the mammalian systems, derived from numerous genetic and biochemical analyses (see Introduction). Based on these findings and greatly facilitated by the sequence of the complete Arabidopsis genome reverse genetic approaches yielded more insights into the various DNA repair mechanisms, pointing to their evolutionary conservation on the level of the protein function [Hays, 2002]. Due to the involvement in the rare events of targeted replacement of genes, a lot of effort was recently put into the elucidation of the homologous recombination pathway, which deals with the repair of double-strand breaks in meiotic and somatic plant cells [Reiss, 2003; Schuermann *et al.*, 2005]. However, knocking-out the Arabidopsis homologues of genes mechanistically implicated in DSB repair by HR or by NHEJ is unlikely to result in novel insights about mechanisms and inter-connections of repair pathways or in the understanding of plant-specific particularities in genome maintenance. In contrast, genetic screens offer a powerful tool to discover novel genes, the products of which are regulating or influencing the relative use of DSB repair pathways; these genes potentially provide new insights into peculiarities of plants in DNA metabolism. In the work presented here, the molecular and phenotypical characterisation of a hyper-recombination mutant is described, which was isolated in a genetic screen for an altered level of HR.

3.1 A novel Arabidopsis HR mutant allele: *hw17*

Two genetic screens in *Arabidopsis thaliana* revealed novel dominant mutations that lead to an alteration in the somatic intra- or inter-molecular recombination frequency, respectively [Molinier *et al.*, in prep]. Among them, the *hw17* mutant allele originated from the screen for altered intra-molecular HR and exhibited an about 3 times higher somatic HRF than wild type plants. The stability and the strength of the hyper-recombination phenotype in *hw17* mutant plants was analysed over several generations as well as in back-crossed populations and was confirmed to be stable over at least 10 generations (Figure 16, page 64). In a hemizygous *hw17* population the number of HR events per plant resembled the standard distribution; the most abundant HR frequency peaked at 3 times more events than in control plants (Figure 17, page 65). Importantly, this distribution analysis is proof of an increased HR frequency in virtually all plants of the hemizygous *hw17* population and excludes the

possibility that the recombination phenotype stems from to a strongly enhanced HR frequency in a small sub-population with secondary mutations or severe homeostatic problems. This suggests a link between the mutated locus and the observed HR phenotype. A direct genetic interaction of the mutated *hw17* and the HR reporter locus can be excluded by the observation of a 5 to 7 times higher HRF monitored in an independent substrate line (Figure 35, page 97).

The molecular analysis of the *hw17* locus revealed that two genes were mutated by the T-DNA insertion (Chapter 2.1.3.1, page 66). The expression of both genes was altered in the hemizygous mutant background; reduced levels were detected for the endogenous full length transcripts whereas truncated mRNAs were more abundant (Chapter 2.1.3.2, pages 68). By genetic means the somatic hyper-recombination phenotype of the *hw17* plants could be assigned to the mutation in the *POLδ1* gene which encodes the Arabidopsis homologue of the catalytic subunit of the eukaryotic DNA polymerase δ. Ectopic expression of the *POLδ1* cDNA in the *hw17* mutant background suppressed the increased somatic HR frequency (Chapter 2.3.2, page 76). The assessment of the HR frequencies in two additional *polδ1* alleles revealed a moderate increase, comparable to that of the *hw17* mutant (Chapter 2.3.1, page 74). In contrast, knock-out plants of the second candidate gene, *RAD26L,* and *hw17* complementation by its cDNA did not yield changes of the HR frequency. These findings clearly prove a causal link between the mutations in the *POLδ1* gene and the increased HR frequencies. The reduced *POLδ1* expression and, presumably, an accordingly lowered POLδ1 protein level resulted in the increased HR phenotype described above (Chapter 2.1.3.2, page 68; Chapter 4.3.3, page 132). Thus, the hyper-recombination *hw17* mutant is renamed *polδ1-1.* The expression of *POLδ1* below a certain threshold level may also explain the dominance of *polδ1* mutations. Indeed, the RNAi-mediated degradation of *POLδ1* transcript correlated with stronger HR phenotypes (Figure 30, page 86). Furthermore, no gross transcriptional changes were detected in *polδ1-1* plants in a genome-wide analysis, suggesting a direct impact of lowered availability of POLδ1 on genome stability (Chapter 2.1.3.2, page 68).

3.2 *Polδ1* alleles alter the intra-molecular HR frequency

The threefold increased intra-molecular HR frequency in *polδ1-1* mutant plants is estimated to be a rather moderate enhancement compared to reported HR induction by DNA damages. On the other hand, also most of the other reported mutations resulting in increased HR exhibit rather weak enhancements, although a direct

comparison with the *polδ1* alleles often cannot be done because of varying time points, modes of HR assessment or recombination substrates. However, moderate increases of the somatic intra-molecular HRF were reported for Arabidopsis plants mutated in *BRU1*, in the checkpoint genes *RAD9* and *RAD17* or for plants over-expressing MIM [Hanin *et al.*, 2000; Heitzeberg *et al.*, 2004; Takeda *et al.*, 2004]. Plants, depleted for the double-strand break sensor *RAD50*, showed a slightly stronger intra-molecular HR phenotype (about 10 fold), assessed in Arabidopsis lines with indirect and direct repeats as substrates for HR (Figure 13, page 60). The latter of them measures also HR events following the SSA model; the comparable enhancement of HR in both substrate lines indicates a channelling of DSB into *RAD51*-dependent and *RAD1*-independent recombination in the *rad50* mutants [Gherbi *et al.*, 2001].

In the Arabidopsis *centrin2* and the tobacco *hyrec* mutants, more pronounced increases in inter-molecular HR are found: there were up to 40 and 1000 times more recombination events, respectively [Gorbunova *et al.*, 2000; Molinier *et al.*, 2004a]. Interestingly enough, the tobacco *hyrec* mutant possesses unique features among the described hyper-recombination plant mutants since it is more resistant to induction of DSBs. Furthermore, its extra-chromosomal HR change is less pronounced and even absent for intra-molecular HR. So far, the report of the *hyrec* mutation, whose molecular nature remains to be elucidated, is the only example showing a diverging alteration of intra- and inter-molecular recombination in somatic plant cells. However, other mutants were not analysed for these aspects. Similarly, the *polδ1-1* mutation resulted in a preferential increase of intra-molecular recombination events between indirect repeats, whereas inter-molecular recombination frequencies remained unchanged (see Chapter 2.5.2, page 96). The differential influence of these two mentioned mutations on intra- or inter-molecular recombination underlines the importance of analysing plant mutants for both types of recombination in the future. These specificities could potentially supply important insights into the reasons why the plant rarely uses HR for DSB repair. Of particular interest may be the examination of Arabidopsis plants over-expressing or being mutated in genes, which code for proteins putatively influencing sister-chromatid cohesion or chromatin composition and structure such as *DDM1*, *MIM* or *BRU1* [Jeddeloh *et al.*, 1999; Mengiste *et al.*, 1999; Hanin *et al.*, 2000; Brzeski and Jerzmanowski, 2003; Takeda *et al.*, 2004].

Apart from the short list of Arabidopsis mutants with an increased HR frequency, to which the *polδ1* mutations can now be added, there are some unmapped mutations or genes known that reduce or even impair recombination [Schuermann *et al.*, 2005].

In the same genetic screen that yielded the *polδ1-1* mutation, an Arabidopsis plant lacking the INO80 ATPase was isolated [Fritsch, 2004; Fritsch *et al.*, 2004]. This protein belongs to the SWF/SNF2 super-family of ATPases and its yeast homologue was shown to be a subunit of a large chromatin remodelling complex, which is involved in the processing of DSBs [Shen *et al.*, 2000; Morrison *et al.*, 2004; van Attikum *et al.*, 2004]. Extra-chromosomal HR of some X-ray sensitive mutants were found to be reduced as well but the corresponding genes were not isolated [Masson and Paszkowski, 1997].

Unfortunately, the somatic HR frequencies of Arabidopsis mutants in known recombination genes are not yet analysed but it is generally assumed that fewer recombination events would be detected. Depletion of proteins that are mechanistically involved in HR essentially leads to the failure of the process. In agreement with this notion, Arabidopsis mutants in these recombination genes exhibit strong phenotypes of meiotic chromosome behaviour due to the failure of DSB repair by HR. In addition, they show enhanced sensitivities to various DNA lesions-inducing treatments (Chapter 1.7.2, page 50). Residual intra-molecular homology-based recombination between repeated sequences may take place in these mutants: recombination according to the SSA model is independent of the HR factors and requires the presence of the NER endonuclease RAD1 [Dubest *et al.*, 2002] (Chapter 1.4.1.2, page 14). The use of the HR substrate with direct repeats, which measures intra- and inter-molecular HR as well as SSA events (Figure 13, page 60), would allow an unbiased estimation of the relative participation of SSA in DSB repair, which was proposed to be the predominant type of HR in somatic plant cells [Gorbunova and Levy, 1999; Puchta, 2005]. HR frequencies of wild type plants should be compared to the residual level of recombination events, detected in HR factors-mutated plants such as RAD51 [Li *et al.*, 2004b]. In budding yeast, SSA is virtually independent of any recombination genes except *Rad52*, of which no homologues could be identified in plants [Paques and Haber, 1999; Reiss, 2003]. The identification of a functional homologue of RAD52 or an analysis of how plants perform HR without it remain the central questions of plant DNA repair and homologous recombination research.

3.3 The DNA Polδ catalytic subunit is highly conserved

Duplication of the eukaryotic nuclear genome is performed by the concerted action of the three replication polymerase complexes α, δ and ε, which are composed of a large catalytic subunit and several accessory proteins [Bell and Dutta, 2002; Hübscher *et al.*,

2002]. The Arabidopsis *POLδ1* gene, the mutation of which is responsible for the HR phenotype of *hw17* plants, encodes the catalytic subunit of the Polδ holoenzyme, as it is evident from the comparison of its amino acid sequence with DNA polymerases of various organisms (Chapter 2.4.1, page 81). With more than 90% sequence similarity, the closest homologue of Arabidopsis POLδ1 is the previously cloned and described rice POLδ1 protein [Uchiyama *et al.*, 2002]. Sequence comparison among the eukaryotic POLδ1 homologues revealed the remarkable similarity of more than 60% for any pair-wise alignment and even bacterial and viral DNA polymerase showed significant sequence homologies to their eukaryotic counterparts. In comparison to other functional protein families, the sequence similarity within the $Pol\delta_{cs}$ homologues from yeast to man is extremely high, suggesting a high conservation also on the structural and functional level. The crucial role of Polδ in replication may not have tolerated many mutations during evolution, underlining the importance of accurate and efficient DNA synthesis for the maintenance of genome integrity.

The Arabidopsis POLδ1 protein contains all highly conserved motifs for the proof-reading and the DNA synthesis activity of $POL\delta_{cs}$ (Chapter 2.4.1, page 81 and Appendix, page XI) [Brautigam and Steitz, 1998; Hübscher *et al.*, 2002]. The N-terminal exonuclease (Exo) domain is responsible for accurate DNA synthesis by its intrinsic proof-reading activity. Indeed, increased spontaneous mutation and tumour formation rates are observed for mutation in a conserved residue in the active site of the exonuclease domain in budding yeast and mice, respectively [Simon *et al.*, 1991; Goldsby *et al.*, 2001]. Recent genetic and biochemical investigations of the Exo domain led to the proposal that this domain must have additional functions in the Okazaki fragment maturation and post-replicative MMR [Jin *et al.*, 2001; Jin *et al.*, 2005]. The C-terminus of Arabidopsis POLδ1 is the least conserved on the amino acid level but the cysteine residues, which form putative zinc fingers, can be found in their typical spatial arrangment (Appendix, page XI). These motifs are also present in Polα and ε and essential for the assembly and function of the Polδ holoenzyme in DNA replication, repair and other cellular mechanisms [Giot *et al.*, 1997; Chanet and Heude, 2003; Sanchez Garcia *et al.*, 2004]. The central protein part of $POL\delta_{cs}$ contains the DNA polymerisation activity and forms the typical DNA polymerase structure resembling the human right hand with palm, thumb and fingers, enclosing and guiding the ssDNA to the catalytic centre [Hübscher *et al.*, 2002]. Mutations in this domain abolish replicative DNA synthesis and exist therefore mostly as temperature-sensitive mutants in budding yeast [Jin *et al.*, 2001; Pavlov *et al.*, 2001].

3.4 The biological functions of Arabidopsis POLδ1

3.4.1 POLδ1 replicates nuclear DNA

The yeast and human Polδ holoenzyme dimerises *in vitro* and is found in a large multiproteinous complex [Maga and Hübscher, 1996; Burgers and Gerik, 1998; Mo *et al.*, 2000; Johansson *et al.*, 2001]. Multiple replication forks are clustered into large replication factories, in each of which a dimeric Polδ complex simultaneously synthesises the leading and the lagging strand, ensuring a coordinated fashion of replication progression (Chapter 1.6.3, page 43) [Waga and Stillman, 1998; Frouin *et al.*, 2003]. Due to this central role of the Polδ holoenzyme in DNA replication and thus in cell division, its largest and catalytic subunit - Pol3 in budding yeast and POLδ1 in most organsisms - is found to be essential for cell proliferation. In agreement with the POLδ1 function in other organisms, for both Arabidopsis *polδ1* alleles, mutated in their coding regions and thus likely to be null alleles, homozygous mutants cannot be obtained (Chapters 2.1.4 and 4.3.1, pages 70 and 129). However, DNA synthesis and the first zygotic cell division are not affected in homozygous Arabidopsis *polδ1-1* embryos (Figure 21, page 72). At this early stage the growth of the homozygous *polδ1-1* arrests; in contrast, the triploid endosperm divides many more times and becomes cellularised similarly to wt endosperms. The further dividing endosperm may be a reason why seed development continues despite of an arrested embryo and abortion takes place relatively late (Figure 20, page 71). The divergent time points of cell division stop in the embryo and endosperm, which are genetically identical but have a different ploidy level, may be explained by varying POLδ1 pools available in the small egg and in the big central cell of the mature embryosac, which are the maternal progenitors of these seed structures. Due to natural decay and dilution effects upon cytokinesis, the depletion of POLδ1 in embryonic cell may happen faster than in the initially multinuclear endosperm. The storage of sufficient amounts of POLδ1 polypeptide, which should be biochemically rather stable, may also explain, how the *polδ1-1* gametes undergo multiple rounds of DNA replication during the haploid phase. Alternatively, other plant DNA polymerases with a comparable processivity and accuracy as Polδ may overtake replication in gametes and in the endosperm.

In accord with the role of Polδ in DNA replication, Arabidopsis *POLδ1* expression is detected more prominently in cells and organs that undergo divisions. *POLδ1* promoter-*GUS* reporter fusions yielded high levels of β-Glucuronidase activity in emerging leaves and in the shoot apical meristem (Chapter 2.4.2, page 83). *POLδ1*

transcripts were found in all tested tissues but at a higher level in the actively dividing cells of an Arabidopsis callus culture. *In silico* analysis for tissue-specific expression of the Arabidopsis transcriptome in a public database basically confirms these findings, showing the strongest *POLδ1* expression levels in callus, suspension cultures, carpels and the shoot apex (https://www.genevestigator.ethz.ch). A similar expression pattern was also reported for the *O.sativa* gene, coding for the POLδ1 homologue [Uchiyama *et al.*, 2002]. *POLδ1* expression in differentiated and not dividing cells accounts for the additional role of Polδ in virtually all DNA repair pathways as well as in endoreduplication (Chapters 1.4 and 1.2.1, pages 10 and 3).

Homozygous *polδ1-1* plants could only be obtained when a transgenic *POLδ1* cDNA over-expressing the protein was supplied. This proved the causal link between the mutation and the cell division phenotype (Chapters 2.3.3 and 0, pages 79 and 125). However, the complemented homozygous *polδ1-1* plants exhibit severe growth and developmental aberrations (Figure 27, page 80; Figure 43, page 127), indicating a partial complementation of the mutation in the *POLδ1* gene, which apparently was not sufficient to restore the biological function of POLδ1. Although the ectopically expressed *POLδ1* mRNA was synthesised to higher levels than the endogenous transcript (Figure 26, page 78), the viral promoter driving the expression of the *POLδ1* cDNA is not strong enough to produce sufficient amounts of POLδ1 in the dividing cells of the meristems (see above). The use of the endogenous promoter to drive the *POLδ1* cDNA may lead to the complete suppression of both the hyper-recombination and the cell division phenotype. However, DNA synthesis presumably is hampered in the shoot apical meristem cells of the complemented homozygous *polδ1-1* plants, leading either to prolongation of the S-phase or, more likely, to cell death (Chapter 1.6.4, page 45). Although plant meristems have the potential to maintain and to restore their integrity, the occurrence of many apoptotic cells may affect plant development and morphology. Indeed, many morphological abnormalities are observed in the complemented homozygous *hw17* plants: misshaped leaves, fasciated stems, loss of apical dominance, aberrant branching and distorted floral organs. These phenotypes resemble the ones observed in other Arabidopsis mutants such as *bru1*, *fas1, fas2* and *msi1*, defective in replication-related processes [Kaya *et al.*, 2001; Hennig *et al.*, 2003; Takeda *et al.*, 2004].

3.4.2 DNA repair synthesis for HR involves POLδ1

Repair of DNA lesions require resynthesis of the removed damaged bases by most of known pathways; exceptions are damage reversion by photolyases and the removal of alkyl residues by alkyltransferases. This final step of repair is performed mostly by the Polδ holoenzyme (Chapter 1.4, pages 10). Elegant *in vivo* studies in budding yeast demonstrated the participation of Polδ in BER, NER and HR during the G1 and G2 phases of the cell cycle [Blank *et al.*, 1994; Budd and Campbell, 1995; Holmes and Haber, 1999; Wang *et al.*, 2004]. Despite its role in repair synthesis, the yeast $POL\delta_{cs}$ depleted strains exhibited no or only weakly increased sensitivities to DNA-damaging treatments (exception: MMS) [Blank *et al.*, 1994; Galli *et al.*, 2003]. Arabidopsis resembles yeast in this respect: a significant change of sensibility to any tested treatment (UV-C, MMS, BLM, CIS, and MMC) was not observed in the *polδ1* mutants (data not shown). In budding yeast and in Arabidopsis as well an enhanced sensitivity phenotype is observed for most of mutants in repair factors. The discrepancy of POLδ1 involvement in DNA repair and the lack of sensitivity may be due to redundant repair synthesis by other DNA polymerases. Alternatively, no sensitivity is observed due to the fact that these tests depend on the inhibition of cell division and thus cannot be separated from the essential function of Polδ1 in DNA replication. However, *polδ1-1* and *polδ1-2* mutant plants partially fail to induce intra-molecular HR to the same extent as wild type control plants upon challenge with various genotoxic compounds (Chapter 2.5.3, page 99). When DSBs were induced with bleomycin in these lines, their HR response was reduced about 7 times compared to wild type plants, indicating a suppression of repair by HR in plants with lower POLδ1 contents. Similar results are also obtained for treatments of *polδ1-1* plants with UV-C and cis-platinum but not for MMS and for treatments of the weaker *polδ1-2* allele. This suppression of intra-molecular HR induction was also reported for temperature-sensitive *pol3-t* budding yeast strains treated with UV and γ-ray. Furthermore, the residual HR induction depended on the presence of RAD52 [Galli *et al.*, 2003]. These findings indirectly imply an involvement of Polδ in DNA repair synthesis, particularly for DSB repair in yeast as well as in Arabidopsis. In respect to the absence of an Arabidopsis RAD52 homologue, it would be interesting to analyse the behaviour of the HR suppression in a *polδ1*/*rad51* or *polδ1*/*rad1* double mutant background, which may indicate whether the residual repair by HR follows the SSA or DSBR model.

3.4.3 A possible role of POLδ1 in organelle maintenance

Apart from a cotyledon-specific hyper-recombination phenotype some of the analysed *POLδ1*-RNAi lines showed a growth inhibition and a chlorotic appearance (Chapter 2.4.3, page 85). Interestingly enough, the dwarfism of these plants does not resemble the developmental phenotype of the partially complemented homozygous *polδ1-1* plants or that of other mutants affected in meristematic DNA replication: shape and number of leaves are normal (see above and Figure 27, page 80). A block of endoreduplication such as reported for Arabidopsis mutants in the B subunit of topoisomerase IV - the *TOP6B* gene - could never be observed in the *POLδ1*-RNAi lines despite of the similarity of the phenotypes [Hartung *et al.*, 2002; Sugimoto-Shirasu *et al.*, 2002]. Endoreduplication takes place in cotyledons of *POLδ1*-RNAi plants and to a lesser extent than in wt plants also in true leaves. This may be due to altered homeostasis in these plants, exhibiting a progressive loss of chlorophyll pigmentation, which presumably results in lowered energy assimilation (data not shown).

Indeed, rosette stage *POLδ1*-RNAi plants show a severe loss of organelles as seen by the comparative analysis of DNA content of the nucleus, mitochondrion and plastids, suggesting a causal relation between reduced POLδ1 levels and amplification or maintenance of organelle genomes (Figure 32, page 90). In mammals, mitochondrial DNA replication and presumably also repair depends on Polγ, of which no clear homologue exists in Arabidopsis [Burgers *et al.*, 2001]. Polγ-like biochemical activity in spinach chloroplasts and two *POLγ*-like rice genes were described, although their biological functions remain to be analysed [Sala *et al.*, 1980; Elo *et al.*, 2003]. However, the reduction of cellular organelle content of some *POLδ1*-RNAi line allows the proposal of the participation of nuclear DNA polymerases in the regulation of organelle replication in plants. Alternatively, the depletion of repair synthesis in *POLδ1*-RNAi plants may cause a degeneration of the organelles, a reduction of their metabolic activity and thus the dwarf phenotype. The concrete mode of action remains to be elucidated. However, it is unclear why this phenotype would be restricted to particular RNAi lines; no clear correlation with *POLδ1* expression and this phenotype can be made (Figure 47, page 134).

3.5 Tentative models for the HR phenotype of *polδ1* plants

3.5.1 Homologous recombination is involved in DNA replication

The increased HR frequencies of Arabidopsis *polδ1* mutants correlated with the lowered *POLδ1* transcript levels proposing a causal link between the replicative DNA synthesis and the hyper-recombination phenotype. For budding yeast strains depleted in DNA replication factors such as Pol30 (PCNA), RFA, Cdc27 (FEN1) and the replication DNA polymerases, genome instabilities ranging from point mutations and micro-satellite instability to gross chromosomal rearrangements were reported [Tran *et al.*, 1995; Chen *et al.*, 1998; Kokoska *et al.*, 1998; Chen and Kolodner, 1999; Chen *et al.*, 1999; Pavlov *et al.*, 2001]. Genetically, these genome instabilities were found to be modulated by the DNA repair genes *Rad1/10*, *Rad50*, *Rad51*, *Rad52*, *Msh3* and *Pms1* indicating an involvement of homologous recombination and mismatch repair in these molecular processes. It is tempting to argue that the above mentioned replication mutants frequently accumulate stalled replication forks, although some of these observed events can be explained by polymerase slippage between short direct repeats [Tran *et al.*, 1995; Kokoska *et al.*, 1998; Kokoska *et al.*, 2000]. The resumption of replication at sites of spontaneously or DNA lesion-induced stalled forks invokes a homology-dependent mechanism that requires HR factors (Figure 39E) [Aguilera, 2001; Michel *et al.*, 2004]. However, a direct evidence for the causality of replication stalling for the genome instability of the Arabidopsis *polδ1* mutant and the yeast replication mutants remains to be shown.

The hyper-recombination phenotypes of Arabidopsis *polδ1* KO mutants showed specificities for intra-molecular recombination between inverted repeats (Chapter 2.5.2, page 96). The same specificity was found when replication was blocked by DNA synthesis inhibitors (Chapter 2.5.1, page 92). Also, the temperature-sensitive yeast strain mutated in *Pol3* (the *pol3-t* allele) behaved in this way [Galli *et al.*, 2003]. In this yeast strain, the frequency of mitotic intra-molecular recombination was assessed with a direct repeat HR substrate and was found to depend more on the Rad1 than on the Rad52 protein. This suggested the prevalent use of the SSA pathway for repair of the molecularly undefined genome destabilisation. In contrast, the induction of intra-molecular HR upon damage-induced replication block in the same *pol3-t* background required Rad52 and not Rad1. Thus, multiple molecular mechanisms may lead to the same phenotypical outcome in this mutant strain. Furthermore, the *pol3-t* mutation was isolated in a genetic screen for enhanced excision of transposable elements flanked by imperfect inverted repeats; this process was found to only depend on the

presence of Rad52 [Gordenin *et al.*, 1992]. These controversial findings suggest that hampered replication can produce a variety of DNA intermediates or structures, which are channelled into multiple molecular pathways that deal with and resolve them. The molecular nature of the replication intermediates as well as the orientation of nearby sequence homologies relative to the direction of replication may influence the outcome of the detected genome alterations.

Stalled replication forks need first to be stabilised in order to avoid the collapsing of the fork and the resulting mutagenic potential. In budding yeast, a central role in replication fork stabilisation is assigned to the RecQ helicase Sgs1 which activates the Rad53 checkpoint, Mec1 and Rad51 [Tercero and Diffley, 2001; Cobb *et al.*, 2003; Bjergbaek *et al.*, 2005]. The molecular mechanism, with which replication resumes at stalled forks, is currently debated and the different proposed models may not be mutually exclusive [Michel *et al.*, 2004]. Hydroxy-urea is generally used to inhibit S-phase DNA synthesis, which results in the stalling of replication forks. In Rad53 checkpoint deficient yeast cells fork reversals were observed whereas such structures have not yet been found in wild type cells. This suggests that they are rather pathological structures, reflecting collapsed forks [Sogo *et al.*, 2002]. However, treatment of Arabidopsis plants with HU resulted in growth reduction, cell cycle arrest [Culligan *et al.*, 2004] and the induction of intra-molecular recombination (Chapter 2.5.1, page 92). In contrast to the *polδ1* KO mutants, DNA synthesis inhibition by HU and by aphidicolin resulted in enhanced intra-molecular HR in a reporter line with direct repeats (data not shown). This implies distinct aberrant replication structures or modes of repair in the genetically or chemically hampered DNA synthesis. Alternatively, the used Arabidopsis reporter line may not have been sensitive enough to measure an increase, although the potential of this line to respond to DSBs by the induction of HR is rather high (data not shown). Enhanced HR between direct repeats was also reported for Arabidopsis plants containing an endogenous replication fork barrier, the non-transcribed spacer region of rDNA repeats [Urawa *et al.*, 2001]. This provided direct evidence for the involvement of HR in replication fork restart in plants.

Arabidopsis *polδ1* KO and RNAi mutants exhibited some striking differences in their HR behaviour. Generally, the enhancement of the HR frequency was rather low in plants hemizygous for a dominant *polδ1* mutation, whereas a very strong increase of HR is observed for most of the *POLδ1*-RNAi lines; this difference cannot be correlated with transcriptional reduction (Chapters 2.3.1 and 2.4.3, pages 74 and 85). Furthermore, an additive effect on the HR level was found in HU-treated plants of a

weak RNAi line but not for the KO mutants, proposing that the former is more susceptible to further replication inhibition (Chapter 2.5.3, page 99).

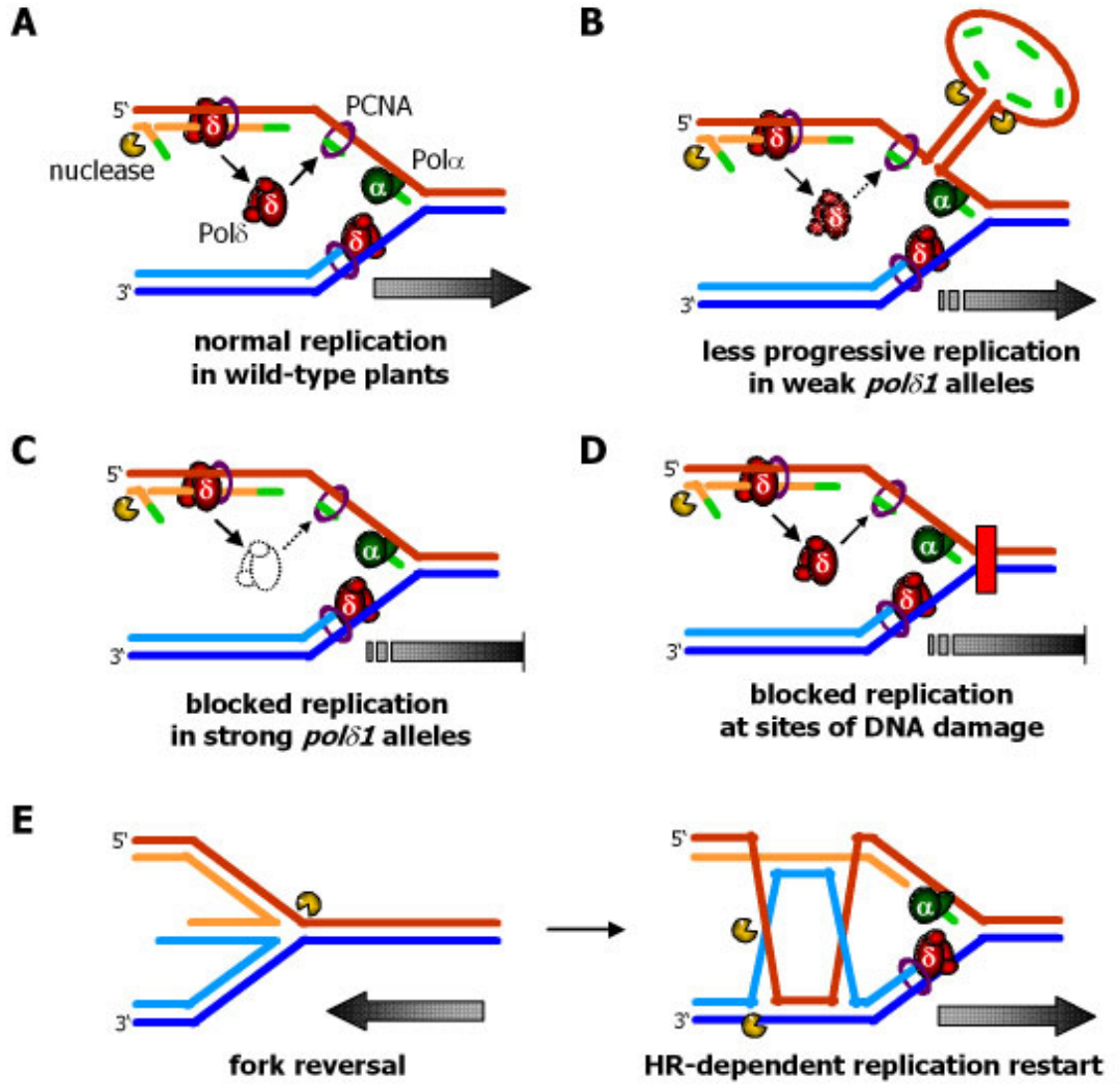


Figure 39: Tentative models for the HR phenotype of *polδ1* plants
A. Scheme of a progressing replication fork in wt plants. **B.** Reduced *POLδ1* expression interferes with replication progression; especially the polymerase switch on the lagging strand may be slowed down. This could lead to the accumulation of unreplicated ssDNA, which is able to form secondary structures. Inverted sequence repeats can form stem-loops that are targets of structure-specific endonucleases, producing DSBs. They are substrates for BIR or post-replicative repair by HR. The stalling of the replication fork may be provoked by severe depletion of functional Polδ (**C**) or by DNA repair intermediates (**D**). The stalled replication forks are channelled into a HR-dependent pathway(s), promoting the restart of replication. (Several models for replication restart are proposed: fork reversal and reassembly of the replication fork is depicted as an example in (**E**).

Preliminary data derived from strong *POLδ1*-RNAi lines crossed into the different HR substrate lines indicated that *POLδ1* knock-down leads to the cotyledon-specific increase of HR, independent on the orientation of the repeats (data not shown). These

findings propose that either the way or the strength of *POLδ1* reduction may trigger the formation of distinct DNA synthesis intermediates. For their resolution or repair they are channelled into different molecular pathways such as BIR or fork reversal, reflecting the distinct HR behaviour of the individual mutant plant.

In conclusion, the hyper-recombination phenotype of *polδ1* mutants may reflect the action of various distinct molecular mechanisms that deal with aberrant DNA intermediates provoked by reduced *POLδ1* expression. Interestingly enough, the *polδ1* KO alleles only exhibited a hyper-recombination phenotype in substrate lines with inverted repeats, which can form a stem-loop structure when single-stranded (Figure 39B). Assuming a slower progression of DNA replication without stalling of the fork in the mutant plants, the lagging strand may be single-stranded for a longer period, leading the accumulation of such stem-loop structures. The removal or processing of the stem-loop may lead to DSBs and thus to the observed HR events.

Similarly, a palindrome of human *Alu* sequences introduced into budding yeast led to closely associated DSB and generation of inverted chromosome duplication [Lobachev *et al.*, 2002]. Inverted repeats in the yeast genome constituted by multiple copies of Ty1 retroelements were found to be frequent breaking points in a yeast strain with lower Polα levels [Lemoine *et al.*, 2005]. A similar model as described above for Arabidopsis *polδ1* mutants was proposed by the authors: lower levels of Polα result in slowed replication and thereby in the formation of cruciform structures promoted by the inverted repeats of the ssDNA at the fork. More evidence for an important role of processive DNA synthesis in chromosome stability is provided by the findings that yeast Mec1 is required to prevent chromosomal breaks in slow replicating zones [Cha and Kleckner, 2002] and that its human counterpart ATR regulates the stability of fragile sites in the chromosome [Casper *et al.*, 2002].

There are some similarities between *polδ1* mutations and the chemically induced stalling of the replication fork in their specificity for intra-molecular HR induction, suggesting that lowered *POLδ1* expression could trigger replication blockage by the lack of sufficient Polδ (Figure 39C). Its restart involves homology-based molecular interaction between the parental and nascent DNA strands at the replication fork [Michel *et al.*, 2004]. In strong *polδ1* alleles, the majority of the observed HR events may originate from blocked replication forks. Alternatively, the frequent stalling of replication forks could arise from incomplete DNA repair in the G1-phase. Reduced *POLδ1* expression may inhibit repair synthesis, leading to the accumulation of repair intermediates (Figure 39D).

3.5.2 Spatiotemporal analysis of the HR events in *polδ1* mutants

In plants, replicative DNA synthesis does not take place solely in the actively dividing cells of meristems but terminal somatic cells also copy their genome without a subsequent mitotic division. This process is called endoreduplication and it results in binominal amplification of the nuclear DNA content (Chapter 1.2.1, page 3). Several lines of evidence suggest that the increased HR frequencies of the *polδ1* allele mainly originate from hampered replication in terminal somatic cells:

A) Due to the multiple clonal divisions of meristematic cells, homologous recombination events are expected to result in cell files or sectors with a functionally restored HR reporter. Such patterns are rarely observed and are not more frequently found in *polδ1* plants (data not shown). In contrast, endoreduplicated cells do not divide and therefore, the restoration of the HR marker during DNA replication leads to the detection of an event in a single cell, which was observed for the majority of HR events in all *polδ1* mutants (Figure 25 and Figure 31, pages 77 and 88).

B) The complementation of the *polδ1-1* mutation by the ectopic expression of the *POLδ1* cDNA suppressed the somatic HR phenotype but only partially corrected the impairment of meristematic replication (Chapters 2.3.2 and 2.3.3, pages 76 and 79). This differential effect of ectopic *POLδ1* expression suggests that these two phenotypes are spatially unlinked. The viral promoter used in the complementation experiment may be strong enough to drive the expression of *POLδ1* cDNA in terminal leaf cells that it suppresses the HR phenotype. In meristem cells the same promoter allows the expression of sufficient *POLδ1* transcripts to overcome the arrest of embryo development; however, this expression level did not allow phenotypically normal growth of the plants (Chapters 2.3.3 and 0, pages 79 and 125). In addition, homozygous *polδ1-1* plants in the HR reporter line 50B complemented with the *POLδ1* cDNA exhibited, apart from the growth phenotype, high luciferase activity, indicating many or clonal HR events (data not shown). Due to technical limitations of the luciferase HR system, it is not possible to determine exactly whether these signals originate from a single or from multiple cells. Therefore, it would be interesting to analyse the complemented *polδ1-1* plants in a *GUS*-based reporter line, which greatly facilitates the localisation of HR events. In these plants one can expected to find more clonal HR events caused by shifting the DNA synthesis impairment from endoreduplication in terminal cells to the meristematic cells, the replication and division problems of which are reflected by the developmental abnormalities.

C) The strong hyper-recombination phenotype of cotyledons of *POLδ1*-RNAi lines was seen neither in embryos before seed maturation nor in germinating seedlings (data not shown). The final number of cells is believed to already be formed at these developmental stages; further growth of the cotyledons depends on the enlargement of the existing cells. The HR events on cotyledons as well as on true leaves appeared only later in development, prevalently in older tissues (Chapter 2.4.3, page 85). Like for the *polδ1* knock-out alleles (see above), the restoration events of the functional HR reporter gene were observed in single cells and were not clonal.

In the hemizygous *polδ1* KO as well as in the RNAi plants, the expression of *POLδ1* was not completely abolished: the steady-state level of *POLδ1* transcript of whole plants was reduced by about 50% (Chapters 2.1.3.2 and 4.3.3, pages 83 and 132). Apparently, this expression level sufficiently supports genome duplication of meristem cells with a 2C nuclear DNA content, allowing normal division and development of the mutant plants. In somatic cells of *polδ1* mutants the lowered expression may limit the pool of available Polδ protein and therefore, lead to a more frequent stalling of DNA replication. These events accumulate with increasing nuclear content of endoreduplicated terminal cells since their requirement for Polδ for additional rounds of genome duplication presumably grows. This may explain the HR enhancement in older leaves of *POLδ1*-RNAi plants or at later developmental stages of *polδ1* KO alleles (Chapters 2.4.3 and 2.5.2, pages 85 and 96).

3.6 Potential application of *polδ1* plants for gene targeting

One of the major challenges of genetic plant engineering still is the precise manipulation of the genomes of higher plants mediated by the HR repair machinery. This targeted modification of the plant DNA appears to be a rare event, estimated to happen about once in 10^3 to 10^5 random transgenesis events [Hanin and Paszkowski, 2003; Schuermann *et al.*, 2005]. In recent years, a lot of basic investigations were undertaken to modulate the efficiency of plants to use HR, yielding a considerable quantity of insights about plant genome maintenance (Chapters 1.4 and 1.8.1, pages 10 and 54). Nevertheless, there are no reports about mutations or methods that lead to the stimulation of the gene targeting frequency, although reliable targeting systems are now available [Hanin *et al.*, 2001; Terada *et al.*, 2002; Reiss, 2003].

It is generally assumed that plants that exhibit an increased level of spontaneous HR may also have an enhanced gene targeting frequency. This assumption does not take into account the possible difference between chromosomal and extra-

chromosomal recombination and between the DNA metabolism of terminal somatic cells and the haploid gametes that are the targets of Arabidopsis transgenesis protocols. Despite of these considerations, a pilot experiment comparing the *polδ1-1* background with wild type plants was performed to evaluate a possible correlation between the increased frequency of somatic recombination and the gene targeting efficiency. In about 8,000 primary transformants, a single targeting event was detected which was not transmitted into the following generation (data not shown). The number of transformants did not allow a statistical analysis and thus a final conclusion. In comparison to the estimated targeting frequency of about 1 in 3,500 transformants for wt plants of the WS ecotype using the same system [Hanin *et al.*, 2001], no substantial enhancement could be assigned to the *polδ1-1* mutation in this experiment. Considering that the increase of somatic HR of *polδ1* mutations is restricted to intra-chromosomal recombination between inverted repeats (Chapter 2.5.2, page 96), it is rather unlikely to find an impact of these mutations on the frequency of gene targeting, which involves recombination between the endogenous target and the extra-chromosomal T-DNA. Furthermore, the essentiality of POLδ1 for various aspect of cellular DNA metabolism would make it difficult to specifically modulate its expression in a way to stimulate targeting efficiency.

Apart from gene targeting, there is another applied aspect of increasing HR frequencies. The genetic separation of closely linked loci is a laborious procedure, which could be shortened by the stimulation of meiotic crossing-over frequency. In fact, the Arabidopsis *xrs* mutants revealed not only altered HR behaviour of somatic cells but also in the frequency of their meiotic crossing-over [Masson and Paszkowski, 1997]. Interestingly enough, in these mutants the alteration of HR frequencies in somatic cells cannot necessarily be correlated to the one in meiotic cells. A mutation in the budding yeast *Pol3* gene coding for the $POL\delta_{cs}$ homologue was found in a genetic screen for deficiencies in meiotic gene conversion [Maloisel *et al.*, 2004]. Apart from a decreased meiotic crossing-over, the gene conversion tracts were also found to be shorter, implying a role for Polδ in the regulation of recombination. Presumably, the mutation conferred lowered Polδ processivity during heteroduplex extension in meiotic HR, resulting in shorter and thus less stable dsDNA intermediates, which could facilitate strand displacement and DSB repair by SDSA without crossing-over. It may be expected that similar mechanisms exist in plants as well. In order to test the influence of the Arabidopsis *polδ1-1* allele on meiotic HR behaviour, a collaboration was initiated

and is still at work, intending to assess genome-wide meiotic crossing-over frequencies by an amplified fragment-length polymorphism procedure (AFLP) [Peters et al., 2001].

3.7 Conclusions and Perspectives

The genome integrity of every organism is safeguarded by the efficiency and accuracy of DNA repair mechanisms. Failed or unfaithful repair of DNA lesions can provoke apoptosis or even cancer in humans [Hoeijmakers, 2001]. However, there is growing evidence that apart from repair, the control and the processivity of replicative DNA synthesis bear an enormous mutagenic potential. In this work it was shown that transcriptional down-regulation of the Arabidopsis *POLδ1*, coding for the catalytic subunit of the major replicative DNA, resulted in genome instability, reflected by an enhanced frequency of homologous recombination. Various alleles of *polδ1* were isolated and characterised, mutants of which showed different strength in HR induction and substrate-specificity for intra- and inter-molecular HR. The *POLδ1* mRNA steady-state level was estimated by semi-quantitative RT-PCR and showed a clear reduction of transcripts for both the T-DNA knock-out and the RNAi. This method may not be accurate enough to demonstrate a correlation between *POLδ1* expression and the strength of the HR phenotype. It would be best to compare cellular POLδ1 protein levels which would also confirm the assumption that the reduced *POLδ1* transcription resulted in lower protein levels. The analysis of the distribution and appearance of HR events in the *POLδ1*-RNAi mutants indicated that the hyper-recombination phenotype originated from endoreduplication in terminal leaf cells. In order to strengthen this link, strong *POLδ1*-RNAi alleles could be used for a careful analysis of the nuclear DNA content and the appearance of the recombination events over time.

The induction of HR in the different substrate lines varied between the hemizygous KO and the RNAi alleles. It was argued that, depending on the strength of the *polδ1* allele, DNA synthesis could have been affected differently, resulting in the formation of distinct replication intermediates. Alternatively, a dose-dependent accumulation of replication intermediates may lead to the involvement of an independent mechanism to rescue replication. In order to elucidate the observed differences between *polδ1* plants in more detail, the HR induction in a cross between the different alleles could be investigated for epistasis or for an additive effect. Alternatively, the repair or replication rescue of the proposed intermediates may require different molecular mechanisms, which depend on the presence of different proteins. HR suppression or stimulation in Arabidopsis double mutants in *POLδ1* and in

the HR-mediating genes such as the *RAD1, RAD51* or the *RecQ-like* [Fidantsef *et al.*, 2000; Hartung *et al.*, 2000; Bagherieh-Najjar *et al.*, 2003; Li *et al.*, 2004a; Li *et al.*, 2004b] could be analysed. Differential dependency would define the importance of the respective molecular mechanism for the observed HR phenotypes in the KO or RNAi-*POLδ1* alleles.

The preferential use of intra-molecular HR in the *polδ1-1* and *polδ1-2* was analogous to chemical replication blockage, proposing that stalling of the replication fork may happen more frequently in *polδ1* mutants. Further evidence for this model was provided by the additive interaction between a weak *polδ1* allele and the replication blocking agent. Resuming replication involves the homology-based molecular interaction between the parental and nascent DNA strands, explaining the hyper-recombination phenotype of these plants. However, another HR reporter construct could be designed in order to strengthen the notion that *polδ1* mutations favour the stalling of DNA replication forks. The natural replication fork barrier of the rDNA repeats can be placed flanking or in-between the repeats for the assessment of intra- and inter-molecular HR [Urawa *et al.*, 2001]. The induction of HR in wild-type plants containing the different substrates can give insights into the molecular mechanisms, which the cell of plants uses to restart replication. The outcome of these experiments can then be compared to the HR behaviour of *polδ1* mutants described above. More importantly, a direct comparison between wild-type and *polδ1* plants in these HR reporter lines with nearby replication blockage can be done: a significant enhancement of the HR is only expected if the *polδ1* mutations also result in more frequent replication stalling.

Apart from increased HR frequencies, budding yeast replication mutants exhibited a variety of other genome alteration, thus they are very likely to be also found in Arabidopsis *polδ1* mutants. Micro- and mini-satellite stability and the frequency of point- or frame-shift mutation can easily be analysed by molecular means [Kovalchuk *et al.*, 2000a; Leonard *et al.*, 2003; Alou *et al.*, 2004]. Such mutations can arise during the process of DNA replication but also from deficiencies in repair synthesis in other phases of the cell cycle. Indeed, the HR frequencies of Arabidopsis *polδ1* mutants were less enhanced than in wild-type plants upon induction of DSBs, underlining an important role of POLδ1 in repair by HR. Reduced levels of POLδ1 may also have an inhibitory effect on repair synthesis by other pathways. This hypothesis could be analysed by *in vitro* repair assays or by immuno-detecting of UV-induced DNA lesions [Liu *et al.*, 2000; Li *et al.*, 2002]. With the latter, the persistence of DNA lesions

upon insult can be assessed as well. DNA lesions or intermediates of failed repair in the *polδ1* mutants could provoke replication blocking when entering into the S-phase. In this model the reduction of *POLδ1* expression would affect replication indirectly but would also result in the same hyper-recombination phenotype.

The high sequence homology of the Arabidopsis POLδ1 protein with other eukaryotic $Pol\delta_{cs}$ as well as the essentiality for cell division implied its conserved function in DNA synthesis. However, a direct effect of the *polδ1* mutation on the DNA replication could not yet be shown. Setting up suspension culture from wild type and *polδ1* mutants may help to investigate the effect of less POLδ1 on DNA replication. The cultures can be synchronised in the G1 phase. After release, initiation time and progression through the S-phase could be assessed in time-laps experiments, revealing possible replication delays.

Chapter 4 Supplementary data

4.1 Detailed characterisation of the *hw17* locus

The plasmid rescue method implied the selection for circularised *Hin*dIII-digested genomic Arabidopsis DNA, which contained the features essential for plasmid replication in *E.coli*. It yielded a plasmid of 5 kb molecular weight, which contained the junction between the T-DNA right border (RB) and the genomic Arabidopsis DNA and corresponded to the *hw17*-specific Southern blot fragment probed for the *Ampicillin Resistance* (*Amp*) gene (Figure 40). The sequenced plasmid contained about 2 kb of Arabidopsis sequence, assigned to BAC clone MBM17 located at the end of the long arm of chromosome V (at 25.6 MB of 26.1 MB). The T-DNA has been integrated into the Arabidopsis gene at5g63950. The single band suggested the presence of a unique T-DNA in the *hw17* locus but probing for the *Sulfonamide Resistance* gene (*Sul*) proximal to the predicted T-DNA left border (LB) revealed a second restriction fragment indicating a duplication of the gene (bands 4a, b). Additional restriction fragment analyses hybridising with both probes supported the presence of only one T-DNA in the *hw17* locus. The duplicated sequence containing the *Sul* gene might originate from rearrangements within the T-DNA or the creation of an additional *Hin*dIII site in the region which is covered by the probe.

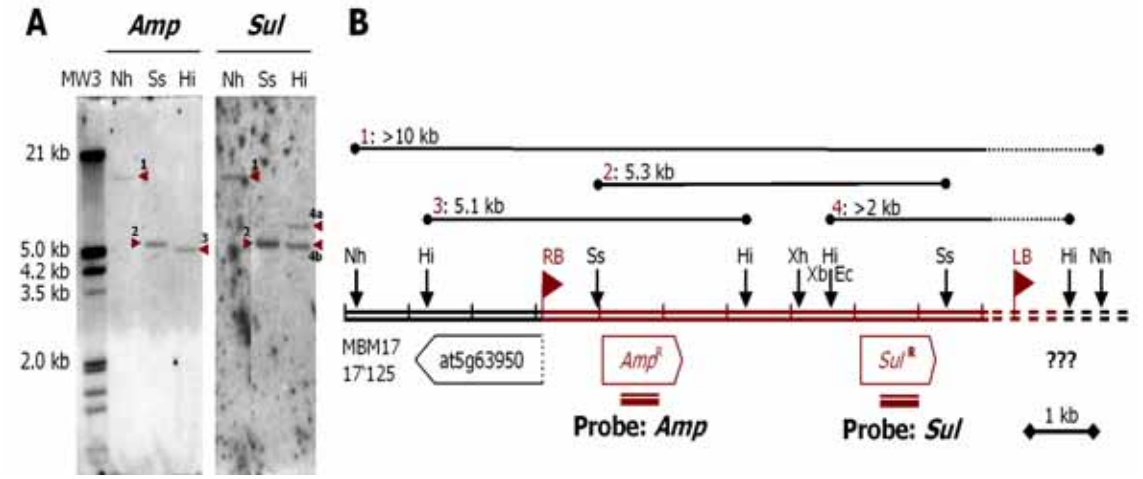


Figure 40: Southern blot analysis of the T-DNA left border junction
A. Southern blotted genomic DNA from hemizygous *hw17* plants was hybridised with a probe for the *Sulfanomide Resistance* (*Sul*) or for the *Ampicillin Resistance* (*Amp*) gene. Control restrictions of wt DNA are not shown. **B.** Map of the mutated locus (black) and the T-DNA (brown). Bars indicate the molecular weight of the Southern bands with the corresponding number. MW3, "DIG molecular weight marker III" (Roche Diagnostics). Restriction enzymes: Nh, *Nhe*I; Ss, *Ssp*I; Hi, *Hin*dIII; Xb, *Xba*I; Xh, *Xho*I; Ec, *Eco*RI.

The isolation of the LB junction turned out to be more difficult. All attempts to amplify the junction by primer walking, inverse PCR and TAIL-PCR failed (data not shown). Southern blot analyses of hemizygous *hw17* plants with probes for the neighbouring genes yielded an altered restriction pattern for the gene at5g63960 - *POLδ1* - (first 4 lanes of Figure 41A) compared to wt plants (data not shown). Starting with outwards primer for this gene, a junction with genomic Arabidopsis DNA was obtained. This sequence was found to be covered by the TAC clone K19M22 which mapped about 2.5 MB closer to the centromeric region of the same chromosome than the *hw17* locus. Extensive Southern and PCR analyses of hemizygous *hw17* plants were performed to confirm this insertion and to understand the molecular composition of this rearrangement (Figure 41 and data not shown). *Hin*dIII (bands 1+9) and *Xba*I (bands 5+17) restriction fragments co-hybridised with probes for the 5' part of the *POLδ1* gene and also for the K19M22 sequence, confirming the fusion of these two genomic regions. By probing for K19M22, the expected restriction fragments of the wt sequence (green) were observed and the stronger hybridisation signal of it compared to the additional one suggested that this genomic region is homozygous.

Probing for the *Sul* gene in the T-DNA, a co-hybridisation of several restriction fragments with the K19M22 probe was found (bands 28+10, 30+12, 31+17). The 5.5 kb *Xba*I fragment even co-hybridised with the 5' *POLδ1* probe (bands 5+17+31), suggesting that the K19M22 sequence insertion might be rather small. Unfortunately, several attempts to obtain the sequence of this mutated region failed. A similar restriction pattern as for the K19M22 probe was seen when the same blot was probed with the 5' sequence of *RAD26L*. These findings favoured a model, which invoked duplication and reinsertion of parts of sequences covered by K19M22 at an ectopic chromosomal position over the possibility of a large genomic deletion and rejoining of the DNA ends.

Figure 41: Southern blot analysis on the rearranged *hw17* locus
A. Southern blotted genomic DNA of hemizygous *hw17* plants was hybridised with a probe for the 5' part of *POLδ1*, the TAC clone K19M22, the 5' part of *RAD26L* and the *Sulfonamide Resistance* gene on the T-DNA. Control restrictions with wt DNA are not shown. **B.** Map of the genomic insertion of TAC clone K19M22 sequences into the *hw17* locus. **C.** Map of the wt locus corresponding to the BAC clone MBM17. **D.** Map of the TAC clone K19M22. Bars indicate the molecular weight of the Southern bands with the corresponding number. Colours mark Southern bands assigned to the insertion of K19M22 sequences into MBM17 (red); to wt fragments of MBM17 (blue); to wt fragments of K19M22 (green); to fragments with hybridising to the T-DNA (brown) and to unassigned restriction fragments (black). MW2, "DIG molecular weight marker II" (Roche Diagnostics). Restriction enzymes: Hi, *Hin*dIII; Ec, *Eco*RI; Xb, *Xba*I; Xh, *Xho*I. (Figure on next page)

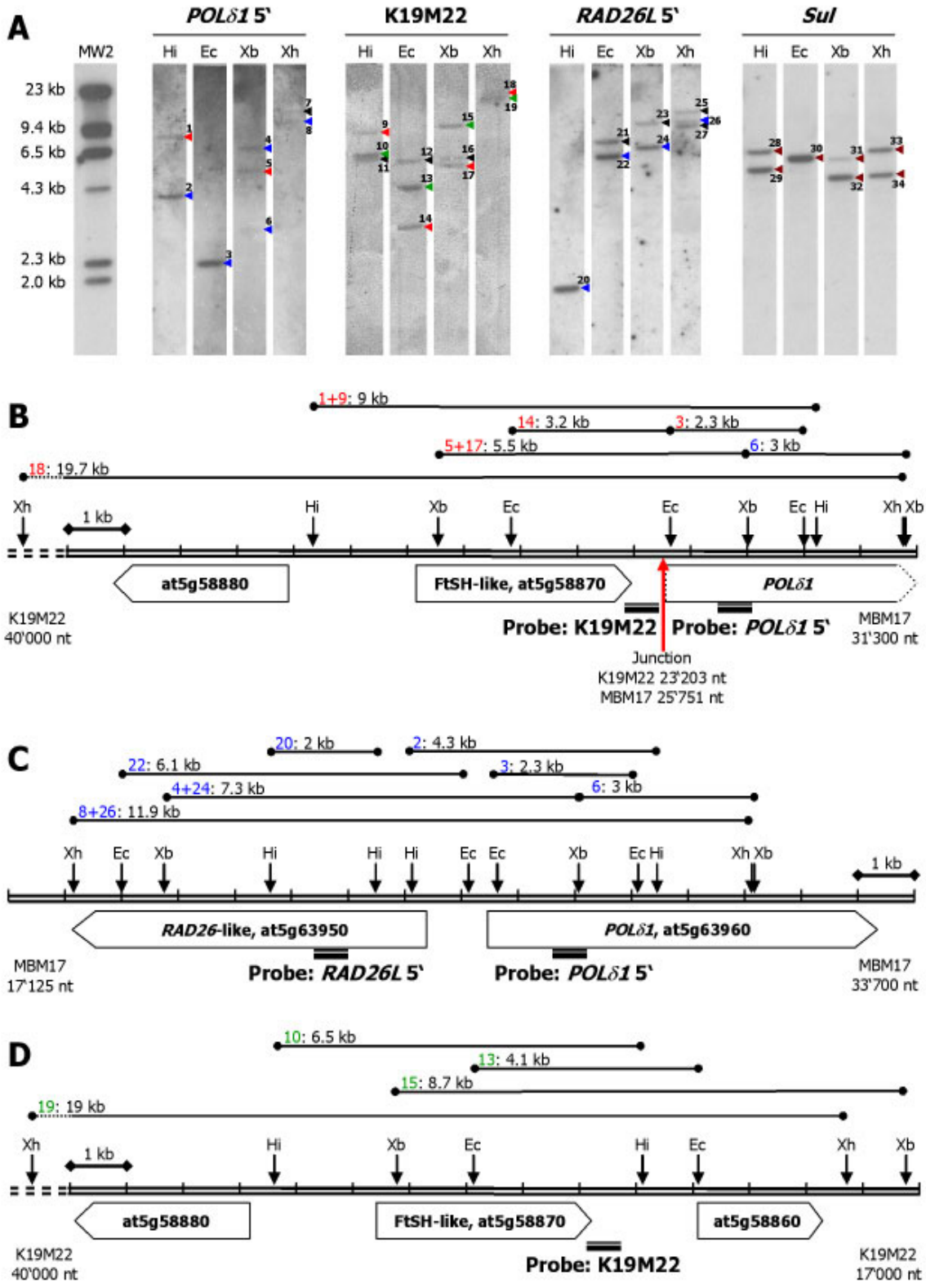


Figure 41: Southern blot analysis on the rearranged *hw17* locus

Although the complex rearrangements of the mutated locus could not be completely solved and many restriction fragments remained unassigned, the obtained molecular data suggested that at least two genes were altered in the *hw17* mutant.

4.2 Complementation of the embryo abortion

In the T2 generation the complemented lines were also analysed for the restoration of a Mendelian segregation of the *hw17* locus independent of their complementation of the HR phenotype. In all control lines and half of the *POLδ1*-complemented lines the ratio between sulfonamide positive and negative plants remained similar to the 2:1 segregation of the untransformed *hw17* family (Figure 42). For the lines 2, 3, 6, 8, and 10 this ratio was clearly increased to the range of the expected 73.3% of resistant seedlings for a recessive zygotic lethal mutation complemented by a single locus T-DNA. This finding indicated that the ectopic expression of *POLδ1* is able to suppress the 2:1 segregation rate caused by the abortive embryo development. The spatial or temporal ectopic expression of the *POLδ1* gene driven by a viral promoter may have limited the rescue to a few families, considering that its mRNA could be detected for all lines but line 14 (Figure 26B, page 78).

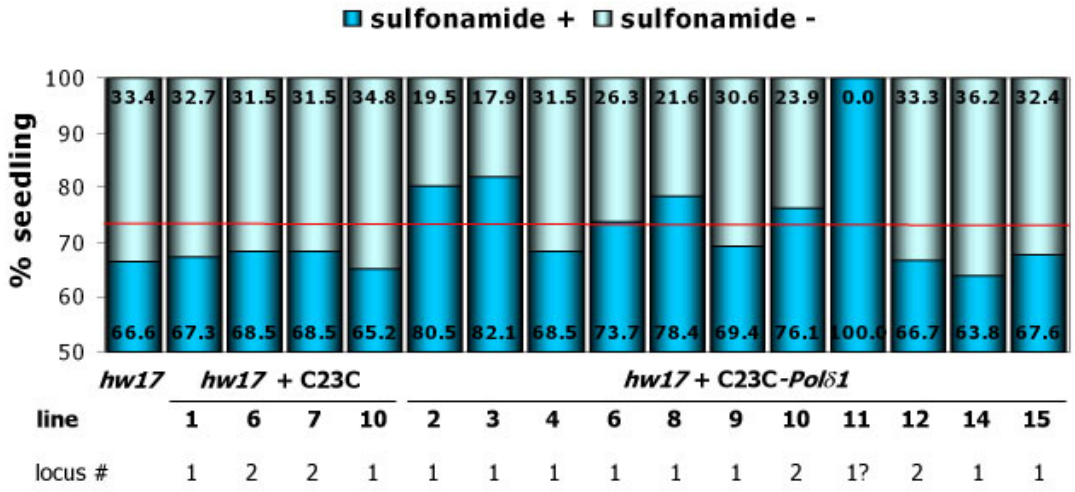


Figure 42: Segregation of the Sulfonamide resistance in complemented lines
T2 families of lines complemented with the vector control or with the *POLδ1* cDNA were assessed for the segregation of the sulfonamide resistance marker in the *hw17* locus and the results were plotted as percentage of the totally assessed seedlings. The loci number of the complementing T-DNA is indicated in the bottom line. The red line reflects the expected ratio between sulfonamide positive/negative plants in a complemented population with one additional copy of the *POLδ1* in an independent locus.

A particular case was found in line 11 in which the *hw17* and the ectopic *POLδ1* T-DNA did not segregate at all, i.e. that all plants were 100% resistant to sulfonamide as well as to kanamycin. This suggested that the T1 plant was already homozygous for the *hw17* locus due to a complementation by several copies of the *POLδ1* cDNA construct which could lead to a nearly 100% kanamycin resistance in the following generation.

However, Southern blot analysis indicated a single T-DNA integration event (data not shown). Since the female gametophytes are the sole targets for the *A.tumefaciens* T-DNA integration [Bechtold *et al.*, 2000; Desfeux *et al.*, 2000], no homozygous plant is expected in the 1st generation. So, it remains to be elucidated what exactly happened in this particular line. The *polδ1-1* allele in line 11 still segregated in the T2 generation indicated by genotyping and the appearance of the dwarf phenotype of homozygous mutants (Figure 43 and Figure 44). A meiotic recombination event in the duplicated region of unknown length between the *rad26l-1* and the *polδ1-1* mutation and the subsequent zygotic fusion with an unrecombined *hw17* gamete could explain this observation. However, a genetic separation of the mutations in the *RAD26L* and the *POLδ1* gene could never be detected in all the attempts to get homozygous *hw17* plants, suggesting that the two alleles are genetically closely linked.

In the previous experiment the number of seedlings was not sufficient to support statistically the restoration of the Mendelian segregation by the *POLδ1* expression. Aiming the isolation of homozygous *hw17* plants, individual plants of lines complemented with *POLδ1* or with the vector control and selected for the *hw17* locus were genotyped by PCR using primer combinations which specifically amplified the mutated or the wt allele. In the complementing lines 3, 8 and 11, in which the restoration of the segregation ratio was indicated in the previous experiment, about one third of the plants were found to be homozygous for the *polδ1-1* mutation as indicated by the absence of the wt PCR band in lane C (Figure 43).

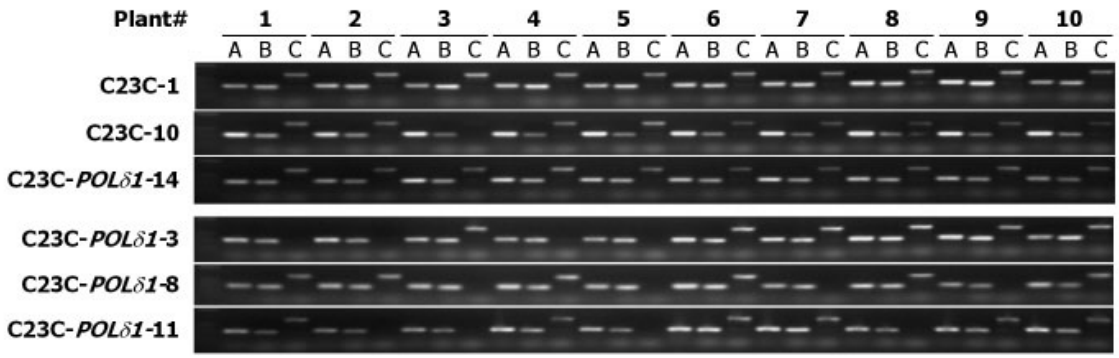


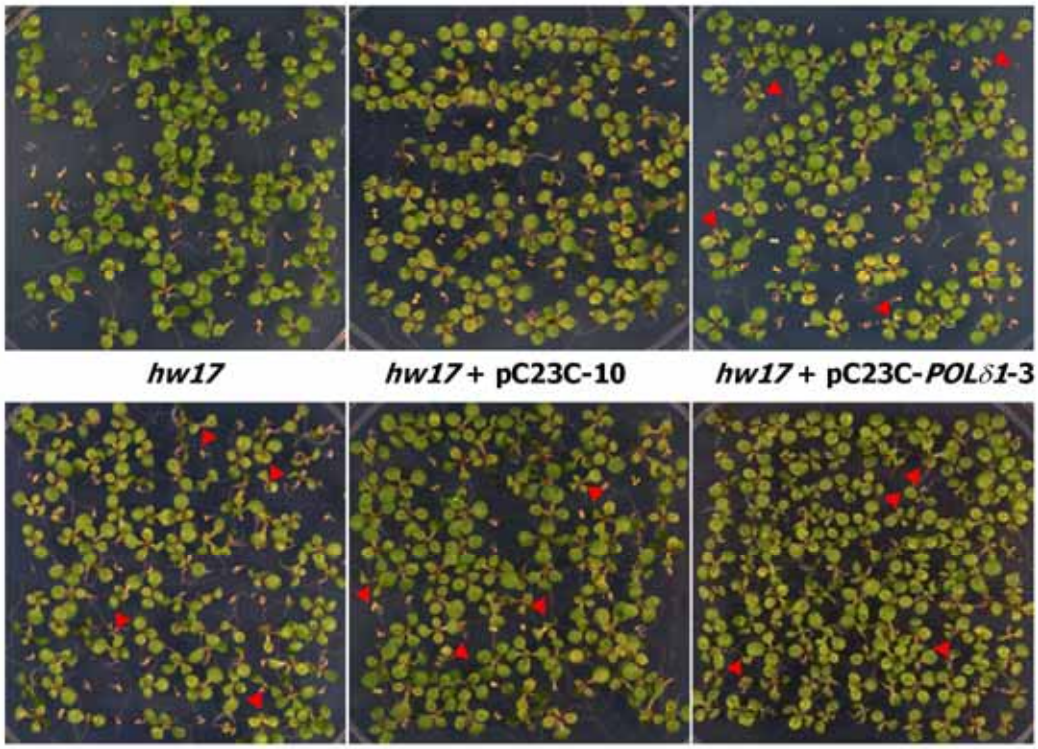
Figure 43: Genotyping of *POLδ1*-complemented lines
Gel electrophoresis of PCR reactions from the genotyping experiment. Six T2 families pre-selected for the *hw17* locus and the complementing T-DNA were scored for the presence of the mutated or the wt allele: the *rad26l-1* (**A**), the *polδ1-1* (**B**) allele and the wt *POLδ1* gene (**C**).

No homozygous plants were isolated in the families complemented with the vector control and also in line 14 in which no ectopic *POLδ1* cDNA could be detected.

Furthermore, in none of the PCR analyses a separation of the *rad26l-1* and the *polδ1-1* allele could be found, suggesting a close genetic linkage of them.

A growth and developmental phenotype emerged from complemented plants identified as homozygous for *polδ1-1*. Whereas cotyledons appeared normal, the subsequent true leaves were abnormally shaped and did not expand properly (Figure 44A). This phenotype resulted in a dwarf appearance and in morphological abnormalities (Figure 27, page 80). This phenotype correlated with the homozygous genotype (Figure 44B).

A



B

resistance phenotype genotype *hw17*	Sul + dwarf -/-	Sul + normal +/-	Sul - dead +/+
hw17		**24**	**12**
C23C-1		20	16
C23C-10		21	15
C23C-*POL1*-14		24	12
control		**65**	**43**

resistance phenotype genotype *hw17*	Sul + dwarf -/-	Sul + normal +/-	Sul - dead +/+
C23C-*POL1*-3	9	17	10
C23C-*POL1*-6	4	24	8
C23C-*POL1*-8	10	18	8
C23C-*POL1*-10	6	18	10
C23C-*POL1*-11	8	16	10
complementing	**24**	**52**	**28**

Figure 44: Correlation of segregation, genotype and phenotype
A. Pictures of the sulfonamide resistance segregation in several complemented and control lines. Red arrow heads indicate homozygous *hw17* seedlings with normally developed cotyledons and typically misshaped and elongated 1st true leaves. **B.** Summary table correlating the restoration of the Mendelian segregation, the zygousity of the *hw17* locus and the observed growth phenotype.

Summarising this data, it could be shown that the *POLδ1* gene and probably the presence of a functional DNA Polδ complex are required for a proper development of the Arabidopsis plants. The complementation of *polδ1-1* driven by a viral promoter did not rescue completely the phenotypes related to the failure of cell division, probably to a spatial or temporal misexpression of the ectopic cDNA.

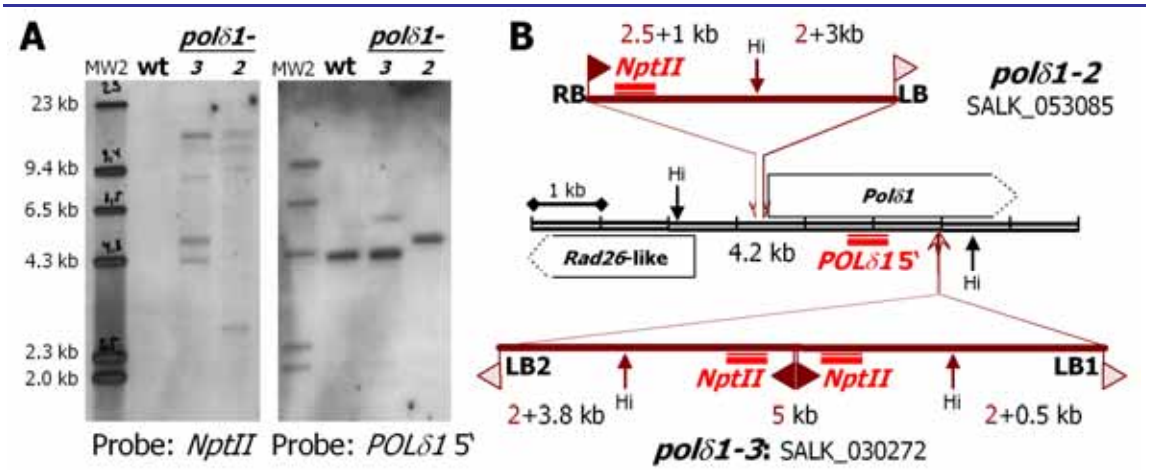
4.3 Characterisation of mutants

All mutant alleles described thereafter were obtained from a T-DNA mutagenised KO collection provided by the SALK Institute. About 100,000 transgenic lines were generated and the insertion of the T-DNA was roughly mapped by a TAIL-PCR strategy with LB specific primers. The obtained junctions were sequenced and annotated in a public database that can be blasted by a web-based interface (http://signal.salk.edu/cgi-bin/tdnaexpress). Mutant alleles for both the *RAD26L* and the *POLδ1* gene were chosen and ordered. Using primers proximal to the T-DNA borders and gene-specific ones, the zygousity of the received T3 plants was evaluated, aiming to obtain and propagate homozygous lines. All mutants were molecularly analysed on the genomic and on the transcription level prior to experimental use.

4.3.1 *Polδ1* alleles

In the lines SALK_053085 and SALK_030272 the T-DNA integration sites were annotated to be in the *POLδ1* gene and therefore named as *polδ1-2* and *polδ1-3*, respectively. With a primer walking strategy the junction sequences on both extremities of the T-DNA were amplified and sequenced (Figure 45C). For the *polδ1-2* allele only the LB junction could be clearly identified, which was found to be in the 5'-UTR of the *POLδ1* gene, 16 bp downstream of the transcription start site and about 67 bp upstream of the translation initiation signal. This insertion was confirmed by Southern blot analysis with a *POLδ1*-specific probe: the 4.2 kb *Hin*dIII fragment of wt plants was absent and instead a predicted fragment of about 5 kb appeared (Figure 45A, B). This also confirmed the homozygousity of this T-DNA mutation. The exact sequence of the 2nd T-DNA junction could not be obtained, probably due to rearrangement or inversions of the adjacent region but some molecular data indicated a deletion of about 600 bp (data not shown). This may correspond to the 3 kb fragment seen on the *Npt*II-probed Southern blot (Figure 45A). On the same blot, at least 4 more bands became apparent, indicating other unmapped T-DNA integration sites in the *polδ1-2* mutant.

For the *polδ1-3* mutation, no homozygous plants could be isolated by PCR-based genotyping. This was in agreement with the Southern blot analysis, in which the 4.2 kb and the additional 5.8 kb restriction fragment corresponded to the wt gene and to the mutant allele, respectively (Figure 45A). Furthermore, also in the *polδ1-3* mutant line several other integrated T-DNAs were found. The strongly hybridising 5 kb fragment of the *NptII*-probed blot could be assigned to a "head-to-head" integration of two T-DNAs in the *polδ1* locus, supported by the isolation of LB sequences on both junctions (Figure 45C). The T-DNA integration events resulted in a deletion of 35 bp of the 10th exon and most likely, a null allele of the *POLδ1* gene was created.



C

Mutant allele: T-DNA border	Junction sequence: T-DNA/ micro homology/ plant sequence	Positon in MBM17 F forward, R reverse
polδ1-2 : RB	n/a	24'970 R ?
polδ1-2 : LB	CAATGTGTTATTAAGTTTCTCTCAGACCTCC	25'653 F
polδ1-3 : LB1	AATGTGTTATTAAGTTGTTGACCTTATTCA	28'123 F
polδ1-3 : LB2	TAAGCGTCAATTTCTTTACTTTCTCTTATTC	28'089 R

Figure 45: The molecular analysis of the *polδ1-2* and *polδ1-3* alleles
A. Southern Blot analysis of *Hin*dIII-digested genomic DNA of wt and of the two *polδ1* alleles probed for the *Kanamycin resistance* (*NptII*) or for the 5' sequence of the *POLδ1* gene (*POLδ1 5'*). MW2, "DIG molecular weight marker II" (Roche Diagnostics). **B.** Schematic map of the locus indicating the position and the orientation of the T-DNA insertion (brown) in the respective mutant alleles. Flags mark the left (LB) and the right (RB) border sequences of the T-DNAs. Hi, indicates the position of the *Hin*dIII restriction sites. Positions of the Southern probes are marked in red. X+Y kb, represent the length of the expected *Hin*dIII restriction fragments composed of T-DNA and genomic sequences. **C**. The table describes the junctions between the T-DNA and the genomic Arabidopsis sequences obtained from both allelic mutants.

No phenotypic alteration in comparison to wt plants were observed for both, the homozygous *polδ1-2* and the hemizygous *polδ1-3* plants. Growth rate, development,

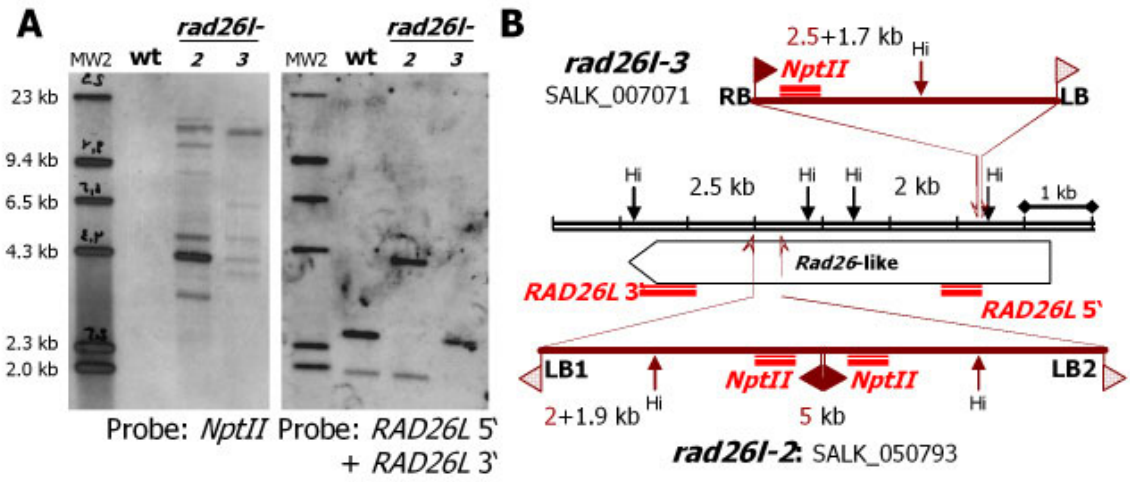
flowering time and fertility were found to be unaffected as well. The fact that no homozygous *polδ1-3* plants could be isolated, suggested an essential function of the POLδ1 protein in plant growth. This supported the findings for the *hw17* mutant in which a homozygous mutation within the ORF of the *POLδ1* gene led to arrested embryo development and seed abortion.

4.3.2 *Rad26l* alleles

In the database, the T-DNA insertion sites of the lines SALK_050793 and SALK_007071 were placed into the coding sequences of the *RAD26L* gene. These two lines were termed *rad26l-2* and *rad26l-3*, respectively. For the *rad26l-2* the two junction sequences between T-DNA LBs and genomic Arabidopsis DNA were obtained (Figure 46C), which were located in the 11th and 13th introns of the *RAD26L* gene. This indicated a deletion of about 600 bp of genomic sequence including the 12th and the 13th exons. Southern blot analysis confirmed these findings; the 2.5 kb wt *Hin*dIII fragment which hybridised with the *RAD26L* 3' probe disappeared and instead a fragment of about 4 kb was observed (Figure 46A, C).

For the *rad26l-3* allele, the T-DNA integration site was found in the 3rd exon of the *RAD26L* gene, producing a small sequence deletion of 32 bp. However, the obtained T-DNA RB junction sequences did not agree with the results from the Southern blot analysis. Instead of an expected band shift from 2 kb to 4.2 kb, which would indicate a *Hin*dIII fragment containing T-DNA and genomic sequences, no band was observed at all (Figure 46A, C). This suggested a more extended deletion of the 5' region of the *RAD26L* gene, which at least covered the sequence corresponding to the *RAD26L* 5' probe. Nevertheless, the integration of the T-DNA in this line was very likely to produce a null allele of the gene since the 5' region of the *RAD26L* gene was substantially altered.

Furthermore, the pattern of the Southern blot analyses confirmed the homozygousity of both *rad26l* alleles and revealed the presence of multiple T-DNA integration sites. One of these additional mutations may have caused the slight growth and the reduced fertility phenotypes that were noted for the *rad26l-3* mutants (data not shown).



C

Mutant allele: T-DNA border	Junction sequence: T-DNA/ filler/ plant sequence	Positon in MBM17 F forward, R reverse
rad26l-2 : LB1	LB sequence-TTAGTTTCTTGCGA	20'249 R
rad26l-2 : LB2	AAGCGTCAATATATTCTGAAACTGAGTC	20'877 F
rad26l-3 : RB	TTTAAACTATCAGTCTATCGGATCTATCT	23'602 R
rad26l-3 : LB	LB sequence-TCAAATTTATCAA	23'634 F

Figure 46: Genomic analysis of the *rad26l-2* and *rad26l-3* alleles
A. Southern Blot analysis of *Hin*dIII-digested genomic DNA of wt and of the two *rad26l* alleles probed for the *Kanamycin resistance* (*NptII*) or for the 5' and 3' sequence of the *RAD26L* gene (*Rad26 5' + 3'*). MW2, "DIG molecular weight marker II" (Roche Diagnostics). **B.** Schematic map of the locus indicating the position and the orientation of the T-DNA insertion (brown) in the respective mutant alleles. Flags mark the left (LB) and the right (RB) border sequences of the T-DNA. Hi, indicates the position of the *Hin*dIII restriction sites. Positions of the Southern probes are marked in red. X+Y kb, represent the length of the expected *Hin*dIII restriction fragments composed of T-DNA and genomic sequences. **C**. The table describes the junctions between the T-DNA and the genomic Arabidopsis sequences obtained from both allelic mutants.

4.3.3 Expression analysis of *POLδ1* and *RAD26l* mutants

Several RNAi knock-down and T-DNA knock-out alleles as well as over-expressing plants for both - *POLδ1* and *RAD26L* - genes were generated or collected from Arabidopsis mutant libraries and used for the functional analysis of their gene products. The effect of the respective transgene on the expression was studied by semi-quantitative RT-PCR.

In the homozygous mutant allele *polδ1-2* in which a T-DNA had integrated into the 5'-UTR region full length *POLδ1* transcripts could not be detected (Figure 47A, D). However, a consistently higher steady-state level of *POLδ1* mRNA was found in RT-PCR reaction covering the 5', central and 3' region of the coding sequences. These findings could explain the vitality of homozygous mutant plants which was never seen for the

other *polδ1* alleles. Although the wild type *POLδ1* mRNA driven by the endogenous promoter was not expressed, a promoter sequence on the T-DNA may produce an aberrant transcript which encodes for POLδ1 or for a mutant polypeptide that can accomplish its biological function. Despite the increased *POLδ1* transcript level in this line, the cell cycle-dependent or spatial regulation of expression was presumably altered and thereby causal for the appearance of the HR phenotype.

In agreement with the T-DNA mutation in one of the parental *POLδ1* copies, the expression of *POLδ1* in the hemizygous line *polδ1-3* was slightly reduced down-stream of the T-DNA integration site (Figure 47A). In the mutated chromosome, the endogenous promoter drove the expression of an aberrant transcript consisting of the 5' region of *POLδ1* and T-DNA sequences. The HR phenotype of *polδ1-3* mutant plants could thus originate from the lowered expression of *POLδ1* or from a putative dominant negative polypeptide encoded by the aberrant transcript.

The expression analysis of both homozygous *rad26l* alleles proved the absence of the full length mRNA encoding for the functional RAD26L protein and suggested they are null alleles (Figure 47B). Residual transcripts were detected up- and downstream of the T-DNA insertion sites, which presumably did not give rise to a functional protein. The ectopic over-expression of a chimeric gene which encoded the functional fusion of the green fluorescence protein (GFP) to the N-terminus of RAD26L was confirmed by RT-PCR (plant line GH4, Figure 47B).

Several Arabidopsis lines were generated in order to reduce the *POLδ1* expression by an RNAi approach. The transcription analysis by RT-PCR confirmed the reduction of *POLδ1* mRNA steady-state levels for most of the lines but not for line c-RNAi 9 (Figure 47C). Interestingly enough, the reduction of *POLδ1* expression level was found to be comparable in i-RNAi and c-RNAi lines although the latter exhibited severe growth inhibition and more pronounced enhancement of HR (see page 90).

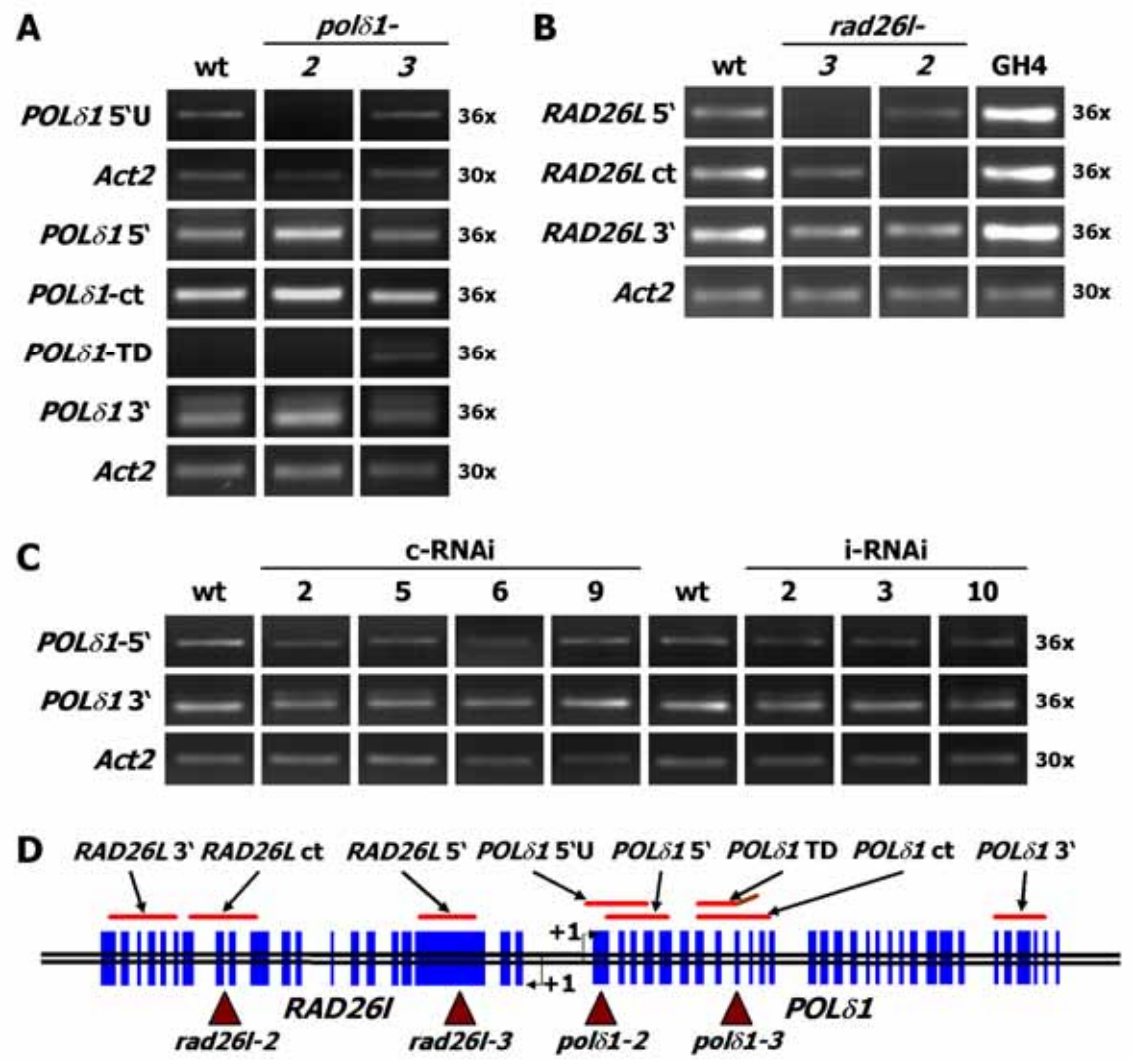


Figure 47: Expression analysis of *POLδ1* and *RAD26l* mutants
Semi-quantitative PCR was done on reverse transcribed total RNA of mutant lines for the *POLδ1* and the *RAD26L* gene. **A.** *POLδ1* steady-state transcript level of the T-DNA KO lines *polδ1-2* and *polδ1-3*. **B.** *RAD26L* steady-state transcript level of the *rad26l-2* and *rad26l-3* KO alleles and line GH4 which ectopically over-expresses a GFP-RAD26L fusion. **C.** *POLδ1* expression levels of chosen constitutive (c) and inducible (i) hairpin RNAi lines. **D.** Scheme of the *hw17* locus, in which the position of the mutations (brown triangles) and of the PCR fragments (red lines) are depicted and the latter are named according to their positions on the transcript. The number of PCR cycles for PCR fragments are indicated in the right column.

Chapter 5 Experimental procedures

5.1 Working with bacteria

Strains and growth conditions

Several *Escherichia coli* and *Agrobacterium tumefaciens* strains for molecular biological applications were used in this work (Table 11). Bacteria were grown either in liquid medium or on solid medium plates. *E.coli* and *A.tumefaciens* cultures were grown in LB or in YEB medium at 37°C or 28°C, respectively (see Appendix: Media, page II).

Organism	Strain	Application	Reference
E.coli	DH5α	Molecular cloning	Invitrogen, San Diego, CA, USA
E.coli	Top10	Plasmid rescue	Invitrogen
E.coli	BL21(DE3) pLysS	Recombinant protein expression	Stratagene, La Jolla, CA, USA
A.tumefaciens	C58C1Rif^R, GV3101	Plant transformation	[Van Larebeke *et al.*, 1974]
A.tumefaciens	A208	Callus formation assay	[Nam *et al.*, 1999]

Table 11: Bacterial strains used in this work

Plasmid mobilisation

For standard cloning procedures, the heat shock transformation method to introduce recombinant plasmids into competent *E.coli* DH5α [Sambrook and Russel, 2001]. The cells were defrosted on ice; the ligation reaction (plasmid) was added and incubated on ice for 15-30 min. After 60 s incubation at 42°C and 5 min on ice, 800 µl LB medium was added. The mixture was incubated for 1 h at 37°C and then plated on the corresponding selective LB medium.

Electro-competent *A.tumefaciens* cells were prepared according to the protocol described elsewhere [Hofgen and Willmitzer, 1988]. 1 µl of plasmid DNA (20-100 ng) and 50 µl of competent cells were transferred into an electroporation cuvette. Electroporation was performed with the GenePulser from BioRad (Hercules, CA, USA) according to the manufacturer's instructions. 800 µl LB medium was added and the mixtures was incubated for 1 h at 28°C, prior to plating onto the corresponding selective growth medium. The same procedure was also used for mobilising plasmids into *E.coli*.

Plasmid extraction from *E.coli*

Depending on the desired yield of plasmid DNA the Mini, Midi or Maxi- Prep Kits from Qiagen (Hombrechtikon, Switzerland) were used.

5.2 Working with plants

5.2.1 Arabidopsis lines

All plant lines used in this study were of the *Arabidopsis thaliana* (L.) Heynh. ecotype *Columbia*-0 (C-0, Col-0). Mutant plants were obtained from a genetic screen [Fritsch, 2004] or from the public Arabidopsis T-DNA knock-out mutant collection at the SALK institute (http://signal.salk.edu/cgi-bin/tdnaexpress) [Alonso *et al.*, 2003].

Intra- and intermolecular homologous recombination events were measured in a series Arabidopsis lines with differently arranged sequence repeats based on the *Gus* or the *Luc* gene. The restoration of the functional reporter gene reflected an HR event according to the respective mechanism (Table 12 and Figure 13, page 60).

Line	HR type	Reporter gene / Repeats	Reference
50B	Intra	*Luc*/ inverted	[Molinier et al., in prep]
1415, 1445	Intra	*Gus*/ inverted	[Gherbi *et al.*, 2001; Fritsch *et al.*, 2004]
1406	Intra/ inter	*Gus*/ direct	[Gherbi *et al.*, 2001]
IC-6C, IC-9C	Inter	*Gus*/ direct	[Molinier et al., 2004b]

Table 12: List of HR substrate lines in an Arabidopsis Col-0 background

5.2.2 Transgenesis

Arabidopsis transformation was achieved by the floral dip method described in [Desfeux *et al.*, 2000]. 3 to 5 plants were grown in pots of 4x4 cm under long day condition for about 3-4 weeks until they had shoots of about 5-10 cm length. Occasionally, flowering was synchronised by cutting back more advanced shoots. Per round of transformation and construct about 120 two 200 plants were prepared.

A.tumefaciens strain C58CIRifR [Van Larebeke *et al.*, 1974] containing the disarmed Ti-plasmid GV3101 and a binary plasmid with the to introducing construct were pre-cultured in 2 ml of YEB for 18 h. The preculture was then used to inoculate a 250 ml culture which was grown for another 18 h. Bacteria were harvested by centrifugation at 4000g for 10 min and resuspended with the infiltration medium IM (10 mM $MgCl_2$, 5% sucrose, 0.05% Extravon® CIBA) of half culture volume and transferred into a 100 ml beaker.

The inflorescences of the plants were dipped into the *A.tumefaciens* solution for few seconds and subsequently kept in a moisture atmosphere for two days. Afterwards, the plants were kept under normal growth condition for another 3 weeks, allowing the maturation of the transformed seeds.

5.2.3 Growth conditions and selection

Depending on the purpose and setup of the experiments, the plant material was either grown *in vitro* on MS agar plates, in soil or germinated *in vitro* prior to a transfer to soil. All seeds were stratified at 4°C in the dark for 2-3 days in order to ensure synchronised germination.

Soil growth condition

Plants were grown on a mixture of two parts of planting soil (GS90 Einheitserde) and 1 part of Perlit supplemented with basic plant fertiliser. The light cycles were set to a 16 h light/ 8 h dark periods at 21°C and 16°C, respectively. 3,500 to 4,000 lux light intensity was provided by Osram L58W BioLux (München, Germany) and Philips TLD36W (Eindhoven, the Netherlands) light bulbs. The relative air humidity was kept at 60 to 70%.

In vitro growth conditons

For *in vitro* culturing of plants, the seeds were surface sterilised by 70% ethanol for 3 min and by 7 % Na-hypochloride, 0.05% Tween-20 for 6-10 min with occasional agitation. After four times rinsing with sterile ddH_2O, the seeds were resuspended in 0.08 % sterile Agar and subsequently spread on MS agar plates. Excess of liquid was removed by air drying in a sterile bench and plates were finally sealed with Micropore™ (3M, St. Paul, MN, USA) surgery tape. Subsequently, the plants were grown under similar conditions as described above.

Selection

Normally, plants were selected for the desired genotype or transgene on MS agar plates supplemented with the respective selective antibiotics or herbicide provided by Sigma-Aldrich (St Louis, MO, USA) or Fluka (Buchs, Switzerland) (Table 13). Seeds were sterilised, plated and germinated like described above. For the selection of primary transformants (T1), the MS agar plates were additionally supplemented with 100mg/l Clavetin® or Timetin®, in order to reduce over-growth by *A.tumefaciens.*

Selection agent	Concentration	Supplier	Plant lines
sulfonamide	10-20 µg/ml	Fluka	*hw17* (*polδ1-1*), c-RNAi lines
kanamycin	50 µg/ml	Sigma-Aldrich	*POLδ1* cDNA complementation
phosphinotricin	15 µg/ml	Sigma-Aldrich	*hw17* (*polδ1-1*), i-RNAi lines
hygromycin	10 µg/ml	Sigma-Aldrich	HR substrate lines
Genotyping by PCR	-	-	*hw17, polδ1-2, polδ1-3, rad26l-2, rad26l-3*

Table 13: List of selective agents for the used plant lines

5.2.4 Plant treatments with genotoxic agents and cell cycle inhibitors

Seeds were sterilised and plated on solid MS as described above, supplemented with the respective selection agent if required. For treatments with chemicals, five days old seedlings were transferred to liquid MS medium and incubated for one day for adaptation. Then, the medium was replaced by liquid MS medium supplemented with the indicated concentration of the respective chemical. For plant sensitivity experiments, the seedlings were incubated for seven to ten days before their fresh weight was assessed. Plantlets were blotted shortly on Whatman paper to remove excess of liquid and groups of 4-8 plantlets were weighted. For HR induction experiments, the genotoxic agents were removed and replaced by liquid MS after two days of incubation. Therein, the plantlets were grown for five more days before they were subjected to HR assessment.

5.2.5 Crossing

Parental plants for crossing were grown individually in long day conditions. As soon as the inflorescence set the first seeds, three to five flowers prior to pollen maturation were emasculated using fine forceps. Remaining flower buds, the inflorescence meristem and opened flowers were removed. The plants with emasculated flowers were kept for another two or three days before the pollen from the paternal crossing partner was applied; this ensured the presence of intact carpels and unfertilised embryosacs. Mature pollen was gently applied onto the sticky stigma of emasculated flower by moving over with an anther. Successful crossing was assessed two days later and plants were grown to seed maturation.

5.3 Basic molecular biology tools

These molecular biological procedures were performed according to standard lab protocol [Sambrook and Russel, 2001] and are only briefly described thereafter.

Analytic plasmid restriction

The restriction enzymes were provided from New England Biolabs (Beverly, MA, USA) and Roche Diagnostics (Rotkreuz, CH). The reaction conditions according to the manufacturer's indication were used but all reactions were supplemented with 100 μg/ml BSA. 200-500 ng of plasmid DNA was used for a reaction volume of 20 μl. The reaction was incubated for 30-60 min. Finally, the restriction fragments were separated by gel electrophoresis (1-2% agarose, TAE buffer) and analysed with the GeneGenius gel imaging system (Syngene, Cambridge, UK).

Cloning

Restriction reactions were basically performed as described above with some modifications. The amount of plasmid DNA, the reaction volume and the incubation time were increased to 1 μg, 60-100 μl and to 2-3 hrs, respectively. A 10-100 fold excess of restriction enzyme split into two doses was add to the reactions to ensure a complete digestion.

Various enzymes were employed to modify the DNA extremities: the T4 DNA polymerase (Roche Diagnostics) to fill in 5'- and to remove 3-protruding ends; the Mung Bean Nuclease (New England Biolabs) to remove 5'-protruding ends; and the Shrimps Alkaline Phosphatase (Roche Diagnostics) to remove 5'-phosphate groups preventing self-ligation of vectors with compatible ends.

The reactions were first check on an analytic gel for complete restriction and the presence of the expected bands (see above) and then separated slowly in an 0.8% low melting agarose gel. The cut bands were melted at 65°C for 10 min with occasional flipping and then directly added to ligation reaction. A visually estimated 3:1 ratio of insert:vector DNA was applied to a 15 μl total reaction mixture prepared according to the provider's instructions. The ligation reaction was incubated over night at 4, 16°C or at RT.

Sequencing

DNA sequencing was performed by the FMI in-house facility, employing a system from PE Applied Biosystems (Foster City, CA, USA) (many thanks to Maciej Pietrzak). They were using a Dye Terminator-based sequencing system. The dRhodamine terminators were introduced into the sequencing template by a "Perkin-Elmer GeneAmp PCR system 9600 or 9700" thermocycler. Finally, the reactions were analysed by an "ABI Prism 3700 DNA sequencer".

5.4 Assessment of the HR frequency

Luciferase-based HR

Plants carrying the luciferase substrate (line 50B) were normally assessed for their recombination frequencies at the rosette stage shortly before bolting. Control and mutant families were arranged in a square of 25x25 cm being a total of 30 to 50 plants. Plants were sprayed with 1 mM luciferin (Biosynth AG, Staad, Switzerland)/ 0.05% Estravon (Novartis, Basle, Switzerland) and incubated in the dark for 30 min in order to allow the uptake and spread of the substrate and to reduce the auto-fluorescence of the chlorophyll. With a highly sensitive liquid nitrogen-cooled CCD

camera in combination with an imaging software package (Gloor Instruments AG, Uster, Switzerland), the light emission of the plants was recorded twice for a 15 min period. Using an arithmetic picture merging method, only signals present on both pictures were extracted and subsequently superimposed to a picture taken in red light. The signals of luciferase activity were assigned to the individual plants and counted.

β-Galactosidase-based HR

Arabidopsis lines carrying a *GUS*-based HR substrate were grown *in vitro* to the indicated developmental stage. The restoration of the functional reporter gene was visualised by histochemical GUS staining according to the standard protocol [Jefferson *et al.*, 1987]. The GUS staining solution consisted of: 100 mM K/Na PO_4 buffer pH 7.0, 10 mM Na-EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1% (v/v) Triton X-100, 1 mg/ml X-Gluc (Biosynth AG). Plants were infiltrated with the GUS staining solution at 100 mbar for 10 min and subsequently incubated at 37°C over night. The chlorophyll of plants was removed by extensive washing with 100% and 70% ethanol on a shaker, prior to visual assessment of the HR events using a stereo-microscope.

Statistical analysis

Luciferase-based HR frequencies were calculated by the number of HR events of a population - counted on the processed picture of an experiment - divided by the number of plants. At least four replica of each experiment (picture) were done and the average HR frequency of the wt control (line 50B) was calculated and used to standardise the individual pictures. The standardised HR frequencies were statistically analysed by the single factor ANOVA function in Microsoft Excel, defining 5% as significance level. Alternatively, the HR events were assigned to the individual plants in a population and statistically analysed by single factor ANOVA, comparing the distribution of HR events between a population of control and mutant plants.

In the *GUS*-based reporter lines, the number of HR events was counted for each individual plant. When the HR frequency was assessed in earlier developmental stages (< 8 true leaves), the average number of HR events per plant was calculated in population of about 40-100 plants for each condition and plant line. The obtained data from at least three independent experiments were statistically analysed by single factor ANOVA, comparing to control plants or conditions. The distribution of HR events in rosette stage plants derived from independent experiments were directly compared with the distribution found for control plants and analysed statistically by ANOVA.

5.5 Extraction methods

Arabidopsis genomic DNA quick preparation for genotyping

Rapid isolation of plant DNA for genotyping by PCR was done according to [Klimyuk *et al.*, 1993]. A small leave piece was collected in an Eppendorf tube and 50 µl of 250 mM NaOH was added. With a pipette tip, the tissue was shortly disrupted and then boiled for 2 min. The mixture was neutralised by adding 50 µl of 250 mM HCl and 30 µl of 500 mM Tris-HCl pH8.0, 0.25 % Triton X-100. After 2 min of boiling and 1 min of full speed centrifugation to remove cell debris, the supernatant of the crude extract was directly subjected to the PCR reaction.

High quality genomic Arabidopsis DNA

Tissue samples were homogenised by grinding in liquid nitrogen using mortar and pistil. Alternatively, the homogenisation was done in a 2 ml Eppendorf tube with two 3 mm tungsten beads and the first buffer of the extraction protocol by vigorous shaking with the Mixer Mill MM300 (Qiagen, Hombrechtikon, Switzerland) for 2 min. The PhytoPure plant DNA extraction kit from "Nucleon™ Phytopure™ Genomic DNA extraction" kit (Amersham Biosciences, Uppsala, Sweden) was employed to extract high quality genomic DNA according to the manufacturer's instructions. Amount and quality of the extracted DNA was assessed by gel electrophoresis and photo-spectrometry.

Plant RNA extraction

Tissue samples were prepared as described above. Total RNA was isolated with the "RNAeasy Plant" kit (Qiagen) following the provider's instructions. In order to remove DNA contamination, the recommended on-column DNase treatment was routinely performed. Quality and quantity of the isolated RNA was analysed by gel electrophoresis and photo-spectrometry.

5.6 PCR-based methods

In recent years, the polymerase chain reaction (PCR) became an important and widely used molecular tool. The PCR technique was used for various applications during this study such as genotyping of plants or *E.coli* colonies, preparation of Southern and Northern probes, molecular cloning, semi-quantitative and circularisation-reverse transcription PCR (RT-PCR, cRT-PCR) (Table 14). A list of amplified PCR fragments for various applications can be found in the Appendix (Page V). DNA polymerases with distinct features were employed depending on the purpose of the experiment and the PCR cycles and reaction buffers were chosen according the supplier's recommendation.

	standard	genotyping	RT-PCR	cloning	DIG probes
Reaction volume	50 µl	25 µl	50 µl	50 µl	50 µl
Primer conc.	800 nM	800 nM	800 nM	1.5 µM	800 nM
dNTP conc.	200 µM	250 µM	200 µM	150 µM	-[7]
Template DNA	-[1]	2 µl[8]	3 µl[3]	50 pg/ 100ng[5]	50 pg/ 100ng[5]
DNA Pol units	1-5[1]	0.5[2]	1[2]	5[6]	2[2]
# of PCR cycles	25-35[1]	35	-[4]	25-30	35

Table 14: PCR reactions for various applications
[1]Various, depending on the purpose of the PCR reaction; [2]Qiagen Taq Polymerase; [3]1/10 diluted reverse transcription reaction; [4]cycle number as indicated, depending on expression level of the assessed gene and the primer combination; [5]plasmid/ genomic DNA as template; [6]Expand™ high fidelity PCR system (Roche Diagnostics) or recombinant Pfu DNA polymerase (Promega, Madison, WI, USA); [7]PCR DIG labelling mix (Roche Diagnostics); genomic DNA, isolated by quick preparation (see Chapter 5.5).

Primers

Primers were designed as follows: GC content of 40-60%, melting temperature of about 58°C in standard salt concentrations, avoiding inverted repeats and repetitive sequences, 21 nt long, G or C at the last two positions. The primers were synthesised by Microsynth (Balgach, Switzerland). A list of the used primers can be found in the Appendix (Page VI).

Semi-quantitative RT-PCR

Expression levels of genes in wt and mutant plants were estimated by the semi-quantitative RT-PCR method. 3 µg of total RNA was reverse transcribed with the "Ready-To-Go™ You-Prime First-Strand Beads" (Amersham Biosciences) supplemented with 5-15 µM oligo-dT primers according to the supplier's indications. The reverse transcription reaction was diluted with 9 volumes of Tris-HCl pH 8.0 and subjected to the PCR reaction as described above. Increasing number of PCR cycles were tested for

each transcript and each primer combination in order to determine the linear range of amplification and the optimal cycle number for quantitative results.

Circularisation RT-PCR (cRT-PCR)

This method allowed the amplification and mapping of the transcription start and the polyadenylisation site with a single RT-PCR reaction and was performed as described previously [Couttet *et al.*, 1997]. 12 μg total RNA derived from wt callus culture was decapped with the tobacco acid pyrophosphatase (Epicentre, Madison, WI, USA) at 37°C for 2 hrs according to the supplier's recommendation. The decapped RNA was circularised with the T4 RNA ligase (Roche Diagnostics) in a 400 μl reaction volume, incubating at 16°C over night. After a chloroform/ ethanol precipitation [Sambrook and Russel, 2001], the concentrated RNA was subjected to "Ready-To-Go™ You-Prime First-Strand Beads" (Amersham Biosciences) and reverse transcription was performed with a *POLδ1*-specific primer (PolF R) according to the manufacturer's instructions. The cDNA containing the 5' and 3' extremities was amplified by two rounds of nested PCR and then sequenced.

5.7 Blotting techniques

The detection of RNA and DNA molecules was performed according to the Northern and Southern blotting protocol described in Sambrook and Russel [2001]. Non-radioactive detection was performed following the supplier's instruction for the "DIG Easy" system (Roche Diagnostics). Accordingly, DIG-labelled molecular weight standards were utilised in parallel to samples.

Northern blotting

10 to 20 μg of total Arabidopsis RNA was denatured by incubating in a MOPS/ formaldehyde/ formamide-containing buffer for 10 min at 65°C and separated according to their molecular weight by gel electrophoresis under denaturing condition (1.2% agarose, 1xMOPS, and 1.8% (v/v) of formaldehyde 37%) running at 80 V. The RNA quality and equal loading was checked visually prior to transfer to a positively charged nylon membrane (Roche Diagnostics). Alternatively, the membrane was stained with methylene blue prior to hybridisation [Herrin and Schmidt, 1988].

Southern blotting

1 μg of genomic Arabidopsis DNA was digested in a 300 μl reaction volume by the appropriate restriction enzymes over night. The restricted DNA was purified by

chloroform/ ethanol precipitation [Sambrook and Russel, 2001] and separated by its molecular weights by gel electrophoresis (0.7% agarose, TAE buffer). The completeness of the restriction and the equal loading were checked visually prior to transfer to a positively charged nylon membrane (Roche Diagnostics).

Hybridisation and detection

DIG-labelled hybridisation probes (of 300 to 600 nt length) for both Northern and Southern blotting were prepared by the "PCR DIG probe synthesis kit" (Roche Diagnostics) according to the supplier's instruction. Membranes were pre-hybridised with "DIG easy Hyb" at 42°C for at least two hours. 2-5 μl of column-purified DIG-labelled PCR product was denatured at 94°C for 10 min and added to 10 ml of hybridisation solution. The hybridisation was done over night at 54°C and 42°C for Northern and Southern blots, respectively.

The membranes were washed twice with 2xSSC/ 0.1% SDS for 10 min and twice with 0.5xSSC/ 0.1% SDS at 68°C for 20 min. Subsequently, the protocol for the chemiluminescence detection by the "DIG Easy" system (Roche Diagnostics) was performed, using the combination of the anti-DIG-AP conjugate antibody and CDP-Star™ (Roche Diagnostics). Finally, the membranes were exposed to Kodak Biomax films.

5.8 Whole genome transcription analysis

5.8.1 Sample preparation

The RNA was isolated from 20-30 two weeks old *in vitro*-grown plants of *hw17* and *rad26l-2* and control lines (50B and Col-0) by the "Plant RNeasy Miniprep" system (Qiagen) according to the manufacturer's instructions. Replicas were prepared analogously with a two weeks interval. 10 μg of total RNA was reverse transcribed by the "SuperScript Choice" system for cDNA synthesis (Invitrogen) according to the protocol recommended by Affymetrix (GeneChip Expression Analysis: Technical Manual (2001) p. 2.1.14-2.1.16) using the 5'-ggccagtgaattgtaatacgactcactatagggaggcgg-t_{24}-3' oligonucleotide. Double-stranded cDNA was cleaned by phenol/ chloroform extraction [Sambrook and Russel, 2001] and by passing the aqueous phase through "Phase-lock Gel" (Eppendorf, Hamburg, Germany). 1 μg of the purified cDNA was *in vitro* transcribed by the "Enzo BioArray High Yield RNA transcript labeling kit" (Enzo Diagnostics, Farmingdale, NY, USA) and purified by the "RNeasy clean-up" columns

(Qiagen) following the manufacturer's protocol. The cRNA was fragmented by heating in 1x fragmentation buffer (40 mM Tris-acetate pH 8.1, 100 mM KOAc, 30 mM MgOAc).

5.8.2 Hybridisation and data analysis

10 µg of fragmented cRNA was hybridised to an ATH1 GeneChip (Affymetrix, Santa Clara, CA, USA) using the standard procedure at 45°C for 16 hours. Washing and staining was performed in a "Fluidics Station 400" (Affymetrix) using the protocol EukGE-WS2v4 and scanned in an Affymetrix GeneChip scanner.

Chip data analysis was performed using the Affymetrix Microarray Suite v5 (target intensity 500 used for chip scaling) and GeneSpring 5.0 (Silicon Genetics). Changes in gene expression were assessed by looking for concordant changes between replicates using a signed Wilcoxon rank test (as recommended by Affymetrix). The "change" *p*-value threshold was < 0.003 for increase and > 0.997 for decrease. After concordance analysis these values become < 9 x10^{-6} and > 0.999991 respectively. Any gene whose detection *p*-value was > 0.05 in all experimental conditions was discarded from the analysis as being unreliable data. Four replicates of the wild type condition and two replicates for each of the mutant plants were done. An Affymetrix change *p*-values for every gene in every pair-wise comparison between the mutant and wild replicates (2 x 4 = 8) for each condition was calculated. A *p*-value threshold of < 0.003 was selected for a significant change. Using set analysis tools, any gene whose direction of change (increase or decrease) was the same in at least 6/8 of the comparisons was retained. The expression values for these highly concordant changes were then assessed and lists of genes with changed expression (increase or decrease) in all alleles tested compared to wild-type were generated.

5.9 Ovule clearing and microscopy

The observation of early embryonic development was performed with a DIC microscope (Carl Zeiss GmbH, Jena, Germany), using whole mounted and cleared ovules according to Herr [1971]. Carpels (0.5-2 mm length) were collected and fixed in FAA (50% ethanol, 5% acetic acid, and 5% formaldehyde) at RT for 20 min, followed by 2 wash steps with 70% ethanol. Fixed samples were dehydrated by consecutive incubation for 20 min in: 70% ethanol, 90% ethanol, 2x 100% ethanol. The ethanol was replaced successively with Herr's buffer (HB: chloral hydrate, lactic acid syrup (85%), Clove oil, phenol, xylene; weight 1:1:1:1:2) by incubating in 20 min intervals with increasing ratio of ethanol/Herr's buffer mixtures (3:1, 1:1, 1:3) and finally twice

with HB. The cleared samples were moved into a drop of HB on a slide and dissected with fine syringe needles. Sufficient HB was added to the sample prior to overlaying with a coverslip.

5.10 FACS analysis

Sample preparation and FACS analysis was basically done as described elsewhere [Galbraith *et al.*, 1983; Barow and Meister, 2003]. 50-100 mg fresh plant tissue was submerged in 1 ml Galbraith buffer (45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate, 0.1% [w/v] Triton X-100) supplemented with 50 μg/ml "DNase free RNase" (Roche Diagnostics) and 50 μg/ml propidium iodide (Sigma-Aldrich) and chopped into small pieces with a razor blade. Alternatively, samples were homogenised with the Mixer Mill MM300 (Qiagen). Subsequently, the homogenate was filtered by passing through a 50 μm nylon mesh.

Nuclear and organelar DNA contents were measured with the FACSCalibur™ flow cytometer (BD Biosciences, Palo Alto, CA, USA) using the 488 nm argon laser and sample flow rate of about 12 μl/min. Propidium iodide fluorescence was excited with about 530 mW at 514 nm and measured in the FL2-channel using a 580/42 nm band-pass filter. The data were analysed with the CellQuest software package.

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Appendix

A Abbreviations

General abbreviations

3meA	3-methyl-adenine
6-4PP	pyrimidine (6-4) pyrimidone photoproducts
8oxoG	8-oxo-guanine
A	adenosine base
ARS	autonomously replicating sequences
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BER	base excision repair
BIR	break-induced replication
BLM	bleomycin
bp	basepair(s)
C	cytosine base
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
CIS	cis-platinum
Col	Arabidopsis ecotype Columbia
CPD	cyclobutane pyrimidine dimers
cs	catalytic subunit
dHJ	double Holliday junction
DNA	deoxyribonucleic acid
DSB	DNA double-strand break
DSBR	double-strand break repair
dsDNA	double-stranded DNA
G	guanine base
gDNA	genomic DNA
GT	gene targeting
HJ	Holliday junction
HR	homologous recombination
HRF	homologous recombination frequency
HU	hydroxy-urea
IC	interstrand crosslink
IR	ionising radiation
kb	kilo basepairs
KO	knock-out
LB	left border sequence of T-DNA
MB	mega basepairs
MMC	mitomycin-C
MMR	mismatch repair
MMS	methyl methanesulfonate
mRNA	messenger RNA
NBS-LRR	nucleotide binding site-leucine rich repeats
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
NIR	nucleotide incision repair
nt	nucleotide(s)
O^6meG	O^6-methyl-guanine
o/n	over night
ORC	origin recognition complex
ORF	open reading frame
ori	bacterial origin of replication
OSI	one-sided invasion
PCR	polymerase chain reaction
Pfr	phytochrome, far red light absorbing
Phy	phytochrome
Pr	phytochrome, red light absorbing
RB	right border sequence of T-DNA
RC	replication complex
rDNA	ribosomal DNA repeats
RIP	repeat induced point mutation
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	reverse transcription
SAM	*S*-adenosylmethionine
SC	synaptonemal complex
SDSA	synthesis-dependent strand annealing
snRNA	small nucleolar RNA
SSA	single-strand annealing
SSB	DNA single-strand break
ssDNA	single-stranded DNA
T	thymidine base
TAC	transformation artificial chromosome
TAIL	thermal asymmetric interlaced
TCR	transcription-coupled repair
T-DNA	transferred DNA
tRNA	transfer RNA
U	uracil base
UTR	untranslated region of cDNA
UV	ultraviolet
UVER	UV-damaged DNA endonuclease-dependent excision repair
V(D)J	variable, diversity, joining
WS	Arabidopsis ecotype Wassilewskija
wt	wild type

Names of genes, proteins and complexes

Amp	ampicilin resistance
ATM	ataxia telangiectasia-mutated
ATR	ATM and Rad3-related
BLM	Bloom syndrome protein
BRCA	breast cancer susceptible
BRU	brushy
Cdc	cell division cycle
CDK	cyclin-dependent kinase
Cdt	Cdc10 target
COP	constitutive photomorphogenic
CS	Cockayne syndrome
DDB	DNA damage binding protein
Ddc	DNA damage checkpoint
Def	RNAPII degradation factor
DET	de-etiolated
Dln	DNA ligase, budding yeast
Dmc	disrupted meiotic cDNA
DNA-PK	DNA-dependent protein kinase
Dpb	DNA polymerase B (II) subunit
ERCC	excision repair cross-complementing
EXO	exonuclease
FEN	flap endonuclease
GUS	β-glucuronidase
HptII	hygromycin phospotransferase
HUS	hydroxyurea sensitive
INO	inositol requiring
KU	thyroid autoantigen
Lif	ligase interacting factor
Lig	DNA ligase, Arabidopsis, human
LUC	firefly luciferase
MCM	mini chromosome maintenance
Mec	mitosis entry checkpoint
MIM	methyl methanesulfonate, ionising radiation and mitomycin-C sensitive
MLH	mutL homologue
MRE	meiotic recombination
MRN	MRE11/RAD50/NBS1 complex
MRX	Mre11/Rad50/Xrs2 complex
MSH	mutS homologue
Mus	MMS and UV sensitive
NBS	Nijmegen breakage syndrome
Nej	non-homologous end-joining defective
NptII	neomycin phospotransferase
PCNA	proliferation cell nuclear antigen complex
PIF	phytochrome interacting factor
PMS	post-meiotic segregation
Pol	DNA/RNA polymerase
RAD	radiation sensitive
Rdh54	Rad54 homologue
RFA	replication factor A
RNR	ribonucleotide reductase
RPA	replication protein A
RFC	replication factor C
RNAP	RNA polymerase holoenzymes
Sgs	slow growth suppressor
SMC	structural maintenance of chromosome
SNF	sucrose non-fermenting
SNM	sensitive to nitrogen mustard
SPO	sporulation deficient
Srs	suppressor of radiation sensitivity
SSB	single-strand binding protein
Sul	sulfonamide resistance
Tel	telomere maintenance
TFIIH	transcription factor IIH
TOP	topoisomerase
UVH	UV hypersensitivity
UVR	UV-B radiation sensitivity
VHL	von Hippel-Lindau syndrome
WRN	Werner syndrome
XP	xeroderma pigmentosa
XRCC	X-ray repair cross-complementing
Xrs	X-ray sensitive

B Media

Bacteria media

LB:	1% (w/v)	bacto-tryptone	YEB:	0.5% (w/v)	beef extract
	0.5% (w/v)	bacto-yeast-extract		0.1% (w/v)	yeast extract
	0.5 % (w/v)	NaCl		0.5% (w/v)	peptone
	1.5% (w/v)	Bacto-agar[1]		0.5% (w/v)	sucrose
				2 mM	$MgSO_4$[2]
				1.5% (w/v)	Bacto-agar[1]

[1] for culture on solid plates
[2] add after autoclaving from 1M stock solution

Plant media

- Standard growth medium: MS macro, MS micro, MS vitamins, 1% (w/v) sucrose, 75 mg/l EDTA, 27 mg/l FeCl3·6H2O, 0.5 g/l 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.7
- Standard growth medium, solid plates: like above, supplemented with 0.8% (w/v) Agar-agar (Merck, Darmstadt, Germany)
- Callus-inducing medium (CIM): MS macro, MS micro, B5 vitamins, 2% (w/v) sucrose, 75 mg/l EDTA, 27 mg/l FeCl3·6H2O, 0.5 g/l MES, 1 mg/l 2,4-dichlorophenoxyacetic acid (2-4D), 0.2 mg/l kinetin, pH 5.7
-

MS macro	1.65 g/l	NH_4NO_3	B5 macro	0.134 g/l	$(NH_4)_2SO_4$
	1.9 g/l	KNO_3		2.5 g/l	KNO_3
	0.44 g/l	$CaCl_2{\cdot}2H_2O$		0.15 g/l	$CaCl_2{\cdot}2H_2O$
	0.37 g/l	$MgSO_4{\cdot}7H_2O$		0.25 g/l	$MgSO_4{\cdot}7H_2O$
	0.17 g/l	KH_2PO_4		0.13 g/l	NaH_2PO_4
MS micro	0.83 mg/l	KI	B5 micro	0.75 g/ml	KI
	6.2 mg/l	H_3BO_3		3 mg/l	H_3BO_3
	16.9 mg/l	$MnSO_4{\cdot}H_2O$		10 mg/l	$MnSO_4{\cdot}H_2O$
	8.6 mg/l	$ZnSO_4{\cdot}7H_2O$		2 mg/l	$ZnSO_4{\cdot}7H_2O$
	0.25 mg/l	$Na_2MoO_4{\cdot}2H_2O$		0.25 mg/l	$Na_2MoO_4{\cdot}2H_2O$
	25 μg/l	$CuSO_4{\cdot}5H_2O$		25 μg/l	$CuSO_4{\cdot}5H_2O$
	25 μg/	$CoCl_2{\cdot}6H_2O$		25 μg/l	$CoCl_2{\cdot}6H_2O$
MS vitamins	0.5 μg/l	Nicotinic acid	B5 vitamins	1 mg/l	Nicotinic acid
	0.5 μg/l	Pyridoxine HCl		1 mg/l	Pyridoxine HCl
	0.1 μg/l	Thiamine HCl		10 mg/l	Thiamine HCl
	20 mg/l	Glycine		100 mg/l	myo-Inositol
	100 mg/l	myo-Inositol			

C Material suppliers

3M	St. Paul, MN, USA	Merck	Darmstadt, D
Affymetrix	Santa Clara, CA, USA	Microsynth	Balgach, CH
Amersham Biosciences	Uppsala, S	New England Biolabs	Beverly, MA, USA
BD Biosciences	Palo Alto, CA, USA	Novartis	Basle, CH
BioRad	Hercules, CA, USA	PE Applied Biosystems	Foster City, CA, USA
Biosynth AG,	Staad, CH	Promega	Madison, WI, USA
Duchefa	Haarlem, NL	Qiagen	Hombrechtikon, CH
Enzo Diagnostics	Farmingdale, NY, USA	Roche Diagnostics	Rotkreuz, CH
Epicentre	Madison, WI, USA	Santa Cruz Biotechnology	Santa Cruz, CA, USA
Eppendorf	Hamburg, D	Sigma-Aldrich	St Louis, MO, USA
Fluka	Buchs, CH	Stratagene	La Jolla, CA, USA
Gibco BRL	Grand Island, NY, USA	Syngene	Cambridge, UK
Gloor Instruments AG	Uster, CH	TaKaRa Bio Inc	Shiga, J
Invitrogen	San Diego, CA, USA		

Table 15: Suppliers of material

D Useful web links

NCBI	http://www.ncbi.nlm.nih.gov/	GeneBank entry page
PubMed	http://www.ncbi.nlm.nih.gov/PubMed	Literature search
TAIR	http://arabidopsis.org/	Arabidopsis database
MIPS	http://mips.gsf.de/	Database for protein and DNA of various organisms
T-DNA express	http://signal.salk.edu/cgi-bin/tdnaexpress	Collection of Arabidopsis mutants and full length cDNAs
ExPASy tools	http://www.expasy.org	Protein analysis tools
ProDom	http://protein.toulouse.inra.fr/prodom/current/html/home.php	Protein domain analysis tool
Microsynth	http://www.microsynth.ch/	Oligonucleotide synthesis and sequencing service
GABI-Kat	http://www.mpiz-koeln.mpg.de/GABI-Kat/db/	Arabidopsis T-DNA mutant database
KAOS	http://www.kazusa.or.jp/kaos/	Arabidopsis genome database
NetGene2	http://www.cbs.dtu.dk/services/NetGene2/	Gene prediction
Genscan	http://genes.mit.edu/GENSCAN.html	Gene prediction
PlantCARE	http://oberon.fvms.ugent.be:8080/PlantCARE/	Plant promoter analysis
Softberry	http://www.softberry.com/berry.phtml	Many tools and links for molecular biology
EMBL-EBI	http://www.ebi.ac.uk/services/	Molecular biology tools and databases
ISREC	http://www.isrec.isb-sib.ch/software/software.html	Protein analysis tools
BIP	http://bip.weizmann.ac.il/toolbox/overview.html	Sequence analysis tools
ChromDB	www.chromdb.org	Plant chromatin database
PlantsP	http://plantsp.genomics.purdue.edu/	Plant protein phosphorylation database
Genevestigator	https://www.genevestigator.ethz.ch/	Arabidopsis micro-array database
PFAM	http://www.sanger.ac.uk/Software/Pfam/	Protein family database

Table 16: List of useful molecular biology databases and tools

E PCR fragments

NAME	PRIMER1	PRIMER2	gDNA (bp)	cDNA (bp)	USE	REFERENZ (page)
Act2	act5' F	act3' R	500	400	R, G	69, 84
Amp	bla5'	bla3'	570	-	D	123
cRT-PCR1 *POLδ1*	PolG F	PolF R	-	880	C	73
cRT-PCR2 *POLδ1*	PolR F	PolO R	-	400	C	73
HptII	hpt 5'	hpt 3'	580	580	R	69
K19M22	K19M22E F	K19M22B R	1250	-	D	124
Luc	luc580 F	luc1270 R	690	690	R	69
NptII	nptII F	nptII R	450	450	D	130, 132
POLδ1 3'UTR	PolR F	anchor	-	340	R	78
POLδ1 5'	PolD F	PolC R	1320	880	D, R	124, 130, 134
POLδ1 5'U	Pol 5'-UTR F	PolV R	960	700	R	69, 134
POLδ1 cDNA	Pol Hind F	Pol Avr R	-	3470	C	73
POLδ1 gDNA 5'	Pol Hind F	PolC R	1400	-	C	73
POLδ1 promoter	proHW2A F	proPOL2A R	1200	-	C	84
polδ1-1 KO (B)	K19M22A	PolF R	350	-	G	127
polδ1-1 wt (C)	IG-HP F	PolF R	660	-	G	127
polδ1-2 LB	Salk LBa1	PolV R	1100	-	G	130
polδ1-2 wt	proHW2A F	PolF R	1370	-	G	-
POLδ1-3'	PolG F	PolH R	590	350/ 420	R	69, 78
polδ1-3 LB1	Salk LBb1	PolI R	600	-	G	130
polδ1-3 LB2	PolL F	Salk LBa1	700	-	G	130
polδ1-3 wt	PolL F	PolI R	900	-	G	-
POLδ1-3'sp	PolG F	PolX R	-	290	R	84
POLδ1-3'us	PolG F	PolW R	450	300	R	84
POLδ1-ct	PolM F	PolT R	1400	430	R	134
POLδ1-TD	PolM F	Salk LBa1	1100	650	R	134
RAD26l 3'	hwhB F	hwhH R	1230	510	R, D	69, 132
RAD26l 5'	hwhM F	hwcD875	630	630	D	124, 132
RAD26L cDNA	Nco hwhe	hwhe Avr	6360	3360	C	73
RAD26l ct	hwcD1956	fp8-3'	1200	580	R	69, 134
rad26l-1 KO (A)	rb-nos#1	fp8-3'	280		G	127
rad26l-1 wt	fp8-5'	fp8-3'	940	-	G	-
rad26l-2 LB1	Salk LBa1	fp8-3'	500	-	G	132
rad26l-2 LB2	hwcD1956	Salk LBa1	420	-	G	132
rad26l-2 wt	fp8-5'	fp8-3'	940	-	G	-
rad26l-3 LB	hwhM F	Salk LBa1	400	-	G	132
rad26l-3 RB	Salk RB1	hwcD875	310	-	G	132
rad26l-3 wt	hwhM F	hwcD875	630	630	G	-
Sul	sul XhoI F	sul XhoI R	1010	1010	D	123

Table 17: List of PCR fragments amplified in the frame of this work
1st column, name or use of PCR amplification; 2nd and 3rd columns, primers; 4th and 5th columns, length of the amplified/ expected PCR fragments; 6th column, type of PCR application, R RT-PCR, G genotyping, D DIG-probe, C cloning; last column, reference to stated use.

F Primers

NAME	SEQUENCE 5'-3'	GENE/REGION
act2-3'	TGTGAACAATCGATGGACCTGAC	*Actin2*
act2-5'	TGGACAAGTCATAACCATCGGAGC	*Actin2*
anchor	GACCACGCGTATCGATGTCGAC	oligo-dT primer
bla3'	AACTTTATCCGCCTCCATCC	*Ampicilin resistance*
bla5'	TTTGCCTTCCTGTTTTTGCT	*Ampicilin resistance*
fp8-3'	TAGTCAGAGTAAGGCCGAGACC	*RAD26l*
fp8-5'	TGTCCATGTGTTCTCTCTTATCC	*RAD26l*
hpt3'	TGCGGAGCATATACGCCCGG	*Hygromycin resistance*
hpt5'	CGGCCGCGCTCCCGATTCCG	*Hygromycin resistance*
hwcD1956	CCTCCAAACTTTCGAAGAAGG	*RAD26l*
hwcD875	TCTATGTGCCTGGTCGTCTTC	*RAD26l*
hwhB F	CTTCTCAAGTTGGTGGTCTCG	*RAD26l*
hwhe Avr	TGCATCCTAGGCATCTTGACTTGTATCTTAC	*RAD26l*
hwhH R	CGCGTCCCAAGAGCAATGCTG	*RAD26l*
hwhM F	AGAGACATCACTAGACAGCGC	*RAD26l*
IG-HP F	TGCCTAATTGTCTTGGTCATG	"intergenic"
K19M22A	AACACATGACACCTCTGGATAC	BAC clone K19M22
K19M22B R	GATGATGTCAACCTAGCTAGC	BAC clone K19M22
K19M22E F	AGTCTAGTGACCATATCATGC	BAC clone K19M22
luc1270 R	GCTATGTCTCCAGAATGTAGC	*Luciferase*
luc580 F	TGCACTGATCATGAACTCCTC	*Luciferase*
M13 reverse	AGCGGATAACAATTTCACACAGGA	pUC, pBSK
Nco hwhe	ACTGTAGCCATGGCGGAAAATAC	*RAD26l*
nptII F	GAGGCTATTCGGCTATGACTG	*Kanamycin resistance*
nptII R	TTGAGCCTGGCGAACAGTTCG	*Kanamycin resistance*
oligo-dT	GACCACGCGTATCGATGTCGAC $(T)_{16}$ (A/C/G)	polyA-tail of mRNA
Pol 5'-UTR F	TTCTCTCAGACCTCCACGAGC	"intergenic"
Pol Avr R	GCTCCCTAGGTACCGTTGTTGCCTGGCTACAG	*POLδ1*
Pol Hind F	GCGCAAGCTTATCCATGGGAAATAGATCCGGTATTTCCAA	*POLδ1*
PolC R	TCCTTTACGACCTGCACACTC	*POLδ1*
PolD F	TGGTGATTCAACACCATCTCC	*POLδ1*
PolF R	ATCTTCGTCGCCGTACAAGAG	*POLδ1*
PolG F	GATCCAATCTACGTGCTACAG	*POLδ1*
PolH R	GGCACTGTGTCCACAGCCTCC	*POLδ1*
PolI R	AAACAGCAAGACGCCTCCTGG	*POLδ1*
PolL F	CTGCTGATGTATGAGATGTATTGG	*POLδ1*
PolM F	CGAAACTTGTTACTCTTCAGG	*POLδ1*
PolO R	CATCATCTAGGGTTCCGATGG	*POLδ1*
PolR F	CACAGTGCCAGGAGTGTCAAG	*POLδ1*
PolS F	TTTGATTCGTGATGTTGATCC	*POLδ1*
PolT R	GAGCTGAGACAGAATTCAGCG	*POLδ1*
PolV R	CAATGCAACTGTAATCTTCAG	*POLδ1*
PolW R	GGCAGCATACCTTGAGACACG	*POLδ1*
PolX R	AGCTCAGCCACTTGAGACACG	*POLδ1*
proHW2A F	CCGCCATGGCTACACCGTTTTCTGGAG	"intergenic"
proPOL2A R	CGCGCCATGGCTTTGGGTGAGCGGGAAA	"intergenic"
rb-nos#1	GGGATCCAGATTGTCGTTTCC	T-DNA pAC102
Salk LBa1	TGGTTCACGTAGTGGGCCATCG	T-DNA pROK2
Salk LBb1	GCGTGGACCGCTTGCTGCAACT	T-DNA pROK2
Salk RB1	CTTCAACGTTGCGGTTCTGTC	T-DNA pROK2
sul XhoI F	TCTCTCGAGTCTACCATGGCTTCTATGATATCCTC	*Sulfonamide resistance*
sul XhoI R	AAACTCGAGCTAGGCATGATCTAACCCTCG	*Sulfonamide resistance*

Table 18: Oligonucleotides used in this work

G Sequences

Sequence 1: cDNA of *POLδ1*

Capital letters indicate translated sequences

```
tagtctcact ttctacttct ctcagacctc cacgagctca caaaatcaca cacattctct tcttttcccg ctcacccaaa
gccATGAATA GATCCGGTAT TTCCAAAAAG CGACCGCCTC CTTCGAATAC CCCACCACCG GCGGGTAAGC ATCGAGCCAC
TGGTGATTCA ACACCATCTC CGGCCATCGG AACCCTAGAT GATGAATTTA TGATGGAAGA GGACGTGTTT CTGGACGAAA
CTCTCTTGTA CGGCGACGAA GATGAGGAAT CCCTAATCCT CCGTGACATT GAGGAGCGTG AATCGCGTTC CTCGGCTTGG
GCTCGACCTC CGCTCTCCCC CGCGTATCTC TCGAATTCAC AGAGTATCAT TTTCCAACAA TTGGAGATTG ACTCTATAAT
CGCGGAGAGT CATAAGGAGC TGTTACCGGG TTCCTCAGGG CAAGCTCCAA TCATTAGGAT GTTTGGGGTT ACCAGAGAAG
GTAACAGTGT GTGTTGCTTT GTTCATGGAT TTGAGCCATA CTTTTACATT GCTTGCCCTC CTGGAATGGG GCCAGACGAT
ATTTCTAATT TCCATCAGAG TCTTGAGGGA AGGATGAGGG AATCCAATAA AAATGCCAAG GTCCCGAAAT TTGTTAAACG
TATAGAAATG GTGCAGAAAA GAAGCATTAT GTATTACCAA CAGCAAAAAT CCCAAACTTT TCTGAAGATT ACAGTTGCAT
TGCCGACTAT GGTGGCAAGC TGTCGCGGCA TCCTTGATAG AGGCCTACAA ATTGATGGAT TGGGTATGAA GAGCTTCCAG
ACATATGAAA GCAATATTCT TTTCGTTCTC CGTTTCATGG TTGATTGTGA TATTGTCGGA GGAAATTGGA TTGAAGTACC
TACTGGGAAG TATAAGAAAA ATGCAAGAAC TTTGTCATAC TGCCAATTGG AGTTCCATTG CCTGTACTCA GATCTAATCA
GTCATGCTGC AGAAGGTGAA TACTCAAAAA TGGCTCCATT CCGTGTACTA AGTTTCGATA TTGAGTGTGC AGGTCGTAAA
GGACATTTTC CGGAAGCTAA GCATGATCCT GTAATCCAGA TAGCGAACCT TGTTACTCTT CAGGGAGAGG ATCACCCATT
TGTACGCAAT GTCATGACTC TTAAGTCATG TGCTCCAATC GTAGGCGTAG ATGTCATGTC TTTTGAAACA GAAAGAGAGG
TCTTACTAGC TTGGAGGGAT TTGATTCGTG ATGTTGATCC TGATATCATC ATTGGTTATA ACATCTGCAA ATTCGATTTA
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TGCAGAGGTC ATTTATGGAG ACACGGATTC AGTCATGGTG CAATTTGGAG TATCGGATGT AGAAGCTGCG ATGACCTTGG
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CTTCTCATTA ACAAGAAGAG GTATGCTGGT TTGCTATGGA CAAATCCTCA ACAGTTTGAC AAAATGGACA CCAAAGGAAT
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Sequence 2: AA sequence of POLδ1

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SNFHQSLEGR MRESNKNAKV PKFVKRIEMV QKRSIMYYQQ QKSQTFLKIT VALPTMVASC RGILDRGLQI DGLGMKSFQT
YESNILFVLR FMVDCDIVGG NWIEVPTGKY KKNARTLSYC QLEFHCLYSD LISHAAEGEY SKMAPFRVLS FDIECAGRKG
HFPEAKHDPV IQIANLVTLQ GEDHPFVRNV MTLKSCAPIV GVDVMSFETE REVLLAWRDL IRDVDPDIII GYNICKFDLP
YLIERAATLG IEEFPLLGRV KNSRVRVRDS TFSSRQQGIR ESKETTIEGR FQFDLIQAIH RDHKLSSYSL NSVSAHFLSE
QKEDVHHSII TDLQNGNAET RRRLAVYCLK DAYLPQRLLD KLMFIYNYVE MARVTGVPIS FLLARGQSIK VLSQLLRKGK
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GETFVKQTLQ KGILPEILEE LLTARKRAKA DLKEAKDPLE KAVLDGRQLA LKISANSVYG FTGATVGQLP CLEISSSVTS
YGRQMIEQTK KLVEDKFTTL GGYQYNAEVI YGDTDSVMVQ FGVSDVEAAM TLGREAAEHI SGTFIKPIKL EFEKVYFPYL
LINKKRYAGL LWTNPQQFDK MDTKGIETVR RDNCLLVKNL VTESLNKILI DRDVPGAAEN VKKTISDLLM NRIDLSLLVI
TKGLTKTGDD YEVKSAHGEL AERMRKRDAA TAPNVGDRVP YVIIKAAKGA KAYERSEDPI YVLQNNIPID PNYYLENQIS
KPLLRIFEPV LKNASKELLH GSHTRSISIT TPSNSGIMKF AKKQLSCVGC KVPISNGTLC ASCKGREAEL YCKNVSQVAE
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```

Sequence 3: Splice variant of *POLδ1*

Capital letters indicate translated sequences

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TGGTGATTCA ACACCATCTC CGGCCATCGG AACCCTAGAT GATGAATTTA TGATGGAAGA GGACGTGTTT CTGGACGAAA
CTCTCTTGTA CGGCGACGAA GATGAGGAAT CCCTAATCCT CCGTGACATT GAGGAGCGTG AATCGCGTTC CTCGGCTTGG
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CGCGGAGAGT CATAAGGAGC TGTTACCGGG TTCCTCAGGG CAAGCTCCAA TCATTAGGAT GTTTGGGGTT ACCAGAGAAG
GTAACAGTGT GTGTTGCTTT GTTCATGGAT TTGAGCCATA CTTTTACATT GCTTGCCCTC CTGGAATGGG GCCAGACGAT
ATTTCTAATT TCCATCAGAG TCTTGAGGGA AGGATGAGGG AATCCAATAA AAATGCCAAG GTCCCGAAAT TTGTTAAACG
TATAGAAATG GTGCAGAAAA GAAGCATTAT GTATTACCAA CAGCAAAAAT CCCAAACTTT TCTGAAGATT ACAGTTGCAT
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ACATATGAAA GCAATATTCT TTTCGTTCTC CGTTTCATGG TTGATTGTGA TATTGTCGGA GGAAATTGGA TTGAAGTACC
TACTGGGAAG TATAAGAAAA ATGCAAGAAC TTTGTCATAC TGCCAATTGG AGTTCCATTG CCTGTACTCA GATCTAATCA
GTCATGCTGC AGAAGGTGAA TACTCAAAAA TGGCTCCATT CCGTGTACTA AGTTTCGATA TTGAGTGTGC AGGTCGTAAA
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aagagaactc ttctcgc

Sequence 4: AA sequence of splice variant of POLδ1

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SNFHQSLEGR MRESNKNAKV PKFVKRIEMV QKRSIMYYQQ QKSQTFLKIT VALPTMVASC RGILDRGLQI DGLGMKSFQT
YESNILFVLR FMVDCDIVGG NWIEVPTGKY KKNARTLSYC QLEFHCLYSD LISHAAEGEY SKMAPFRVLS FDIECAGRKG
HFPEAKHDPV IQIANLVTLQ GEDHPFVRNV MTLKSCAPIV GVDVMSFETE REVLLAWRDL IRDVDPDIII GYNICKFDLP
YLIERAATLG IEEFPLLGRV KNSRVRVRDS TFSSRQQGIR ESKETTIEGR FQFDLIQAIH RDHKLSSYSL NSVSAHFLSE
QKEDVHHSII TDLQNGNAET RRRLAVYCLK DAYLPQRLLD KLMFIYNYVE MARVTGVPIS FLLARGQSIK VLSQLLRKGK
QKNLVLPNAK QSGSEQGTYE GATVLEARTG FYEKPIATLD FASLYPSIMM AYNLCYCTLV TPEDVRKLNL PPEHVTKTPS
GETFVKQTLQ KGILPEILEE LLTARKRAKA DLKEAKDPLE KAVLDGRQLA LKISANSVYG FTGATVGQLP CLEISSSVTS
YGRQMIEQTK KLVEDKFTTL GGYQYNAEVI YGDTDSVMVQ FGVSDVEAAM TLGREAAEHI SGTFIKPIKL EFEKVYFPYL
LINKKRYAGL LWTNPQQFDK MDTKGIETVR RDNCLLVKNL VTESLNKILI DRDVPGAAEN VKKTISDLLM NRIDLSLLVI
TKGLTKTGDD YEVKSAHGEL AERMRKRDAA TAPNVGDRVP YVIIKAAKGA KAYERSEDPI YVLQNNIPID PNYYLENQIS
KPLLRIFEPV LKNASKELLH GSHTRSISIT TPSNSGIMKF AKKQLSCVGC KVPISNGTLC ASCKGREAEL YCKNVSQGML
PFFPFL

Sequence 5: *POLδ1* fragment used for RNAi constructs

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TGATAGAGAT GTTCCAGGGG CAGCTGAAAA TGTCAAGAAA ACCATTTCGG ATCTTCTCAT GAACCGTATT GACTTGTCAC
TTTTGGTGAT TACTAAGGGT CTAACGAAAA CAGGAGATGA TTATGAAGTT AAATCAGCTC ATGGTGAACT TGCTGAACGC
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CAAGGCTTAT GAACGATCAG AAGATCCAAT CTACGTGCTA CAGAATAATA TCCCTATAGA CCCAAATTAC TACTTGGAGA
ATCAGATTAG CAAGCCACTT CTT
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Sequence 6: Chimeric cDNA used for complementation

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ATGGGAAATA GATCCGGTAT TTCCAAAAAG CGACCGCCTC CTTCGAATAC CCCACCACCG GCGGGTAAGC ATCGAGCCAC
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Sequence 7: cDNA of *RAD26L*

Capital letters indicate translated sequences

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AGTATCCTCC AGGATCTTTC TGCGCCTCCT AGACAGCCTC CCTCTTCTTC TCATGGAGAA GATGAAGAGA CGAAGAAGTC
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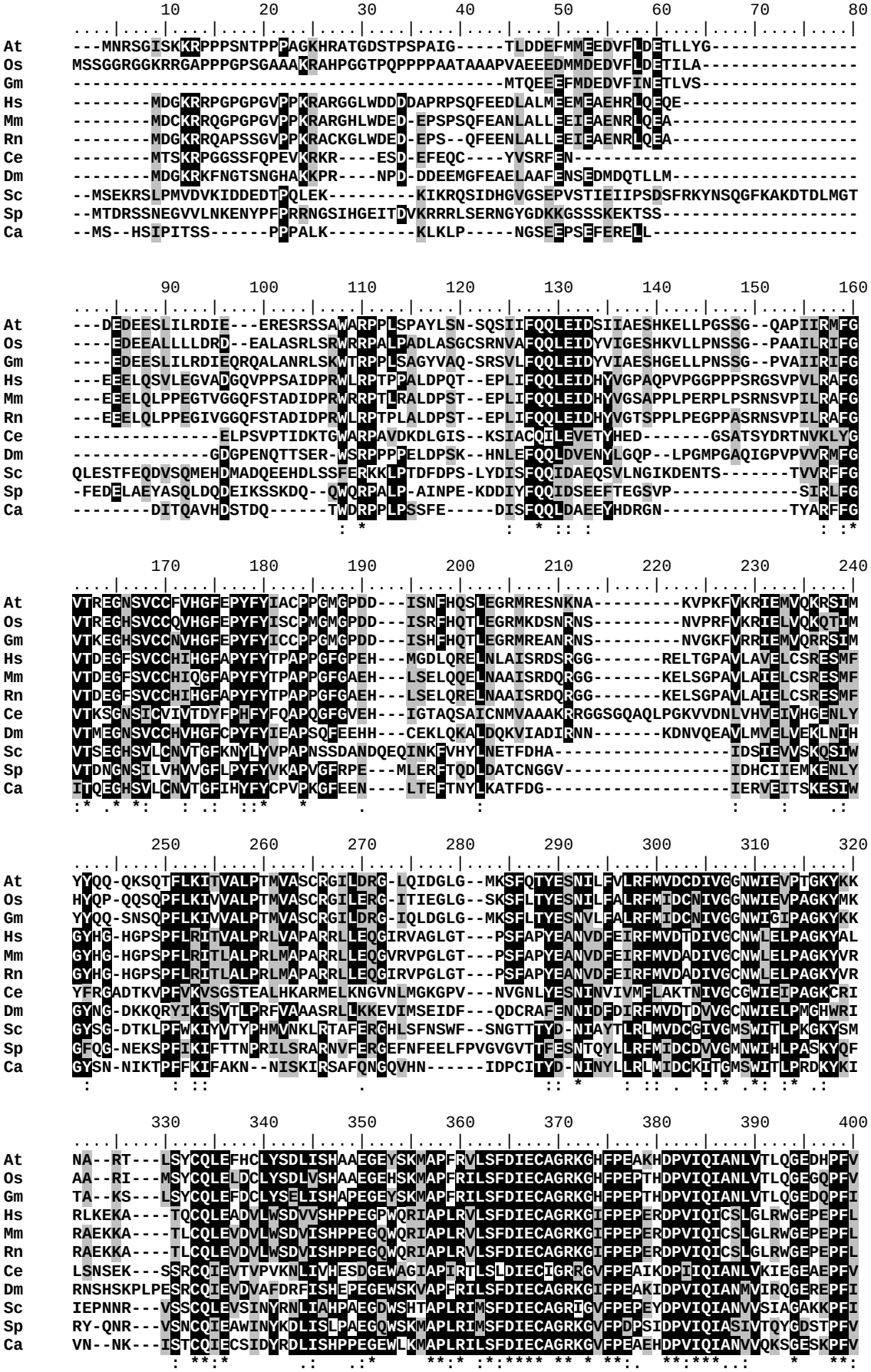
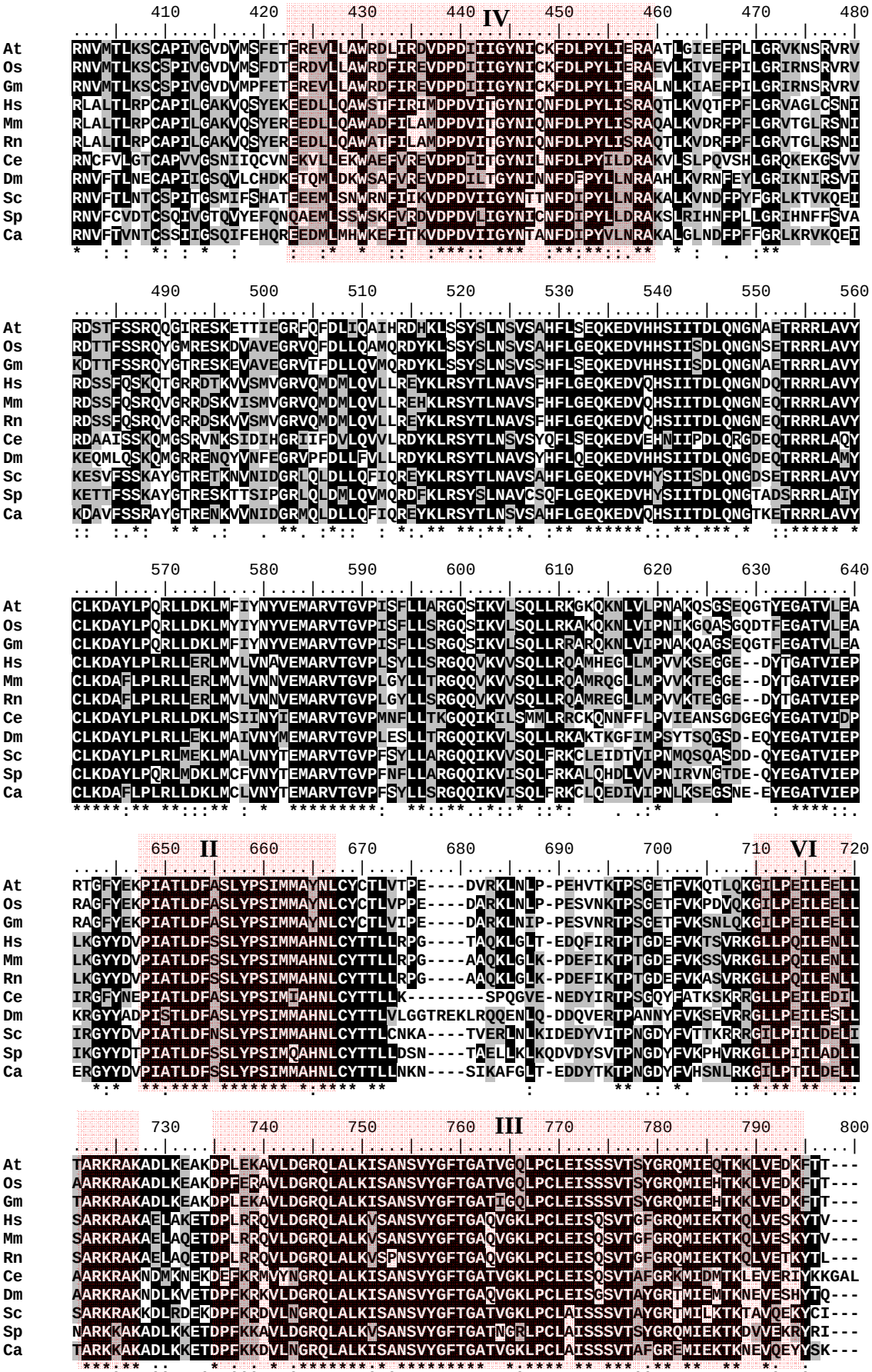
CTCCTATTCA AGCGATACAG AAAGATGAAG AAGAACAAAT AAGGAGAGAA ACAGCATTGC TCTTGGGACG CGCATCAGCA
AGTATTTCAC AAGACACCGT CATCAATGGG GCTGACTATG CTTTCAAGCC AAAGGATGTG AATTTGGACA AGAGAATCAA
CATTTCCCCA GTCGATGACA AGGAATTGTC AGAAAGCGTA ATTAAAGCAA GACTCAATCG TTTGACGATG CTATTACAAA
ACAAGGGTAC GGTCTCAAGG CTACCTGATG GAGGGGCAAA AATCCAGAAG CAGATTGCTG AATTGACTCG AGAACTGAAA
GACATGAAAG CAGCAGAAAG GATCAACATG CCTCAAGTTA TTGACTTGGA GGAGGATATA AGTCGGAAGA TGCAAAAAGG
ATTGAATCTG TAGagtaaga tacaagtcaa gatgcaagaa atgcaaacga ccatcattgc aacacttgtg gttttttttt
gttccttatc taatttggtt tggttgaatt ggtaagtcaa ttaccatatg acttgctgca aaggagtgtg cgaagccaga
ttttatcttt tctcaaaccc aaactttaat ccgcggtaaa atgaagattt cagcacac

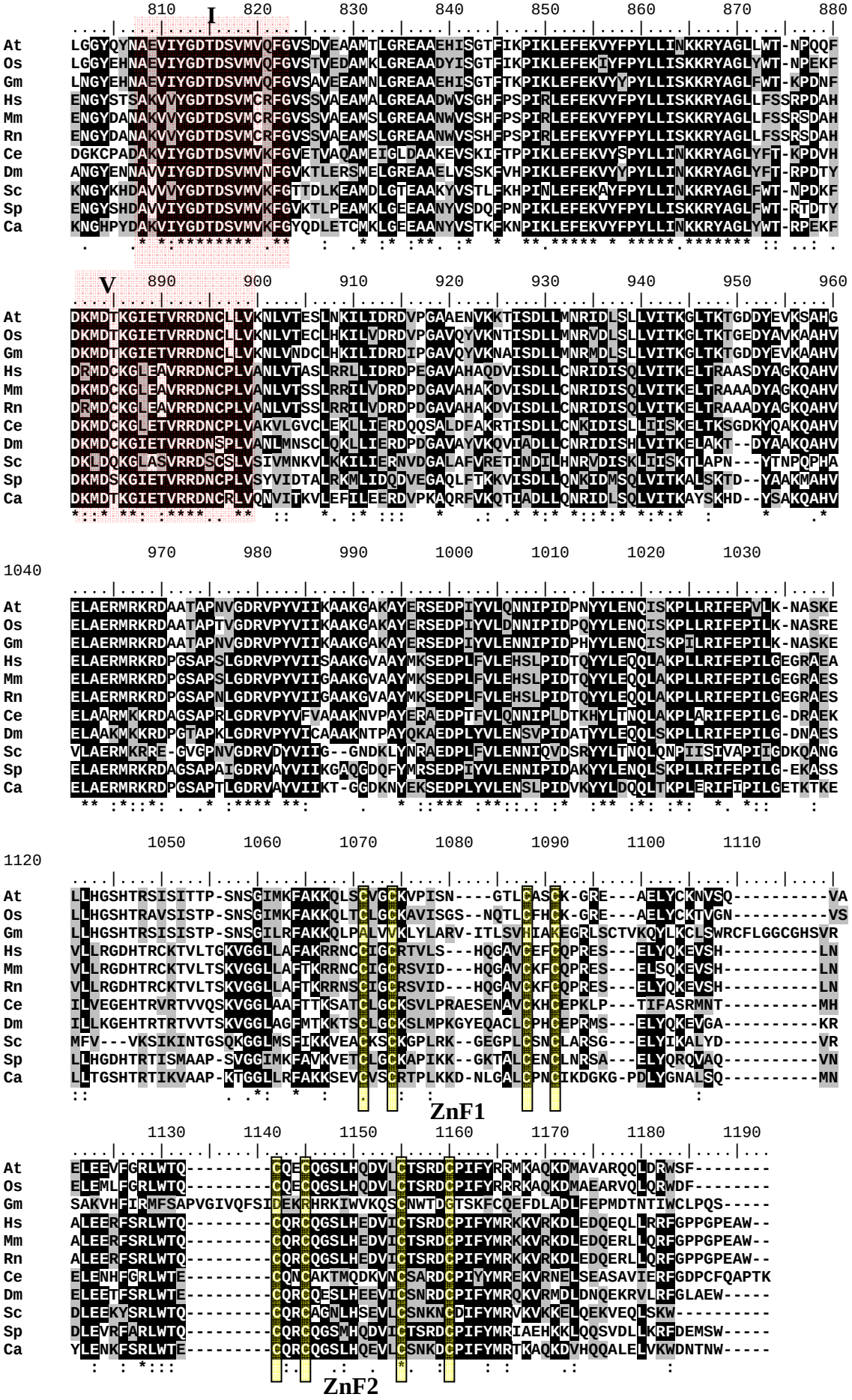
Sequence 8: AA sequence of *RAD26L*

MAENTASHRR KPRSLNDRHY SILQDLSAPP RQPPSSSHGE DEETKKSMIK LAGRRRLCKA LPKEDEADGY DDPDLVDFYS
PVKGETSLDS AGIGNKFTSW DESKEANTEL AGEPNFSIIT DFCSPSPQLK QKEEMQGDGG RNEIMGILDD LTSKLGTMSI
QKKKDSQSND FDACGVKSQV DKFDFEDAKS SFSLLSDLSK SSPDVVTTYN AGVNSIKDKQ GKSGFAIREE QTSKEFSREW
EERISNVGKQ NSYSGRHFDD NSEDNRQGYN LDRGKSQCKE VDQSMKTTRH IEVSEKIRTV GRSNAAKLRD LDEDDDDDDC
LILSGKKAAE MKINKPARSY NAKRHGYDER SLEDEGSITL TGLNLSYTLP GKIATMLYPH QREGLNWLWS LHTQGKGGIL
GDDMGLGKTM QICSFLAGLF HSKLIKRALV VAPKTLLPHW MKELATVGLS QMTREYYGTS TKAREYDLHH ILQGKGILLT
TYDIVRNNTK ALQGDDHYTD EDDEDGNKWD YMILDEGHLI KNPNTQRAKS LLEIPSSHRI IISGTPIQNN LKELWALFNF
SCPGLLGDKN WFKQNYEHYI LRGTDKNATD REQRIGSTVA KNLREHIQPF FLRRLKSEVF GDDGATSKLS KKDEIVVWLR
LTACQRQLYE AFLNSEIVLS AFDGSPLAAL TILKKICDHP LLLTKRAAED VLEGMDSTLT QEEAGVAERL AMHIADNVDT
DDFQTKNDSI SCKLSFIMSL LENLIPEGHR VLIFSQTRKM LNLIQDSLTS NGYSFLRIDG TTKAPDRLKT VEEFQEGHVA
PIFLLTSQVG GLGLTLTKAD RVIVVDPAWN PSTDNQSVDR AYRIGQTKDV IVYRLMTSAT VEEKIYRKQV YKGGLFKTAT
EHKEQIRYFS QQDLRELFSL PKGGFDVSPT QQQLYEEHYN QIKLDEKLES HVKFLETLGI AGVSHHSLLF SKTAPIQAIQ
KDEEEQIRRE TALLLGRASA SISQDTVING ADYAFKPKDV NLDKRINISP VDDKELSESV IKARLNRLTM LLQNKGTVSR
LPDGGAKIQK QIAELTRELK DMKAAERINM PQVIDLEEDI SRKMQKGLNL

Sequences 9: Alignment of eukaryotic catalytic subunits of the POLδ



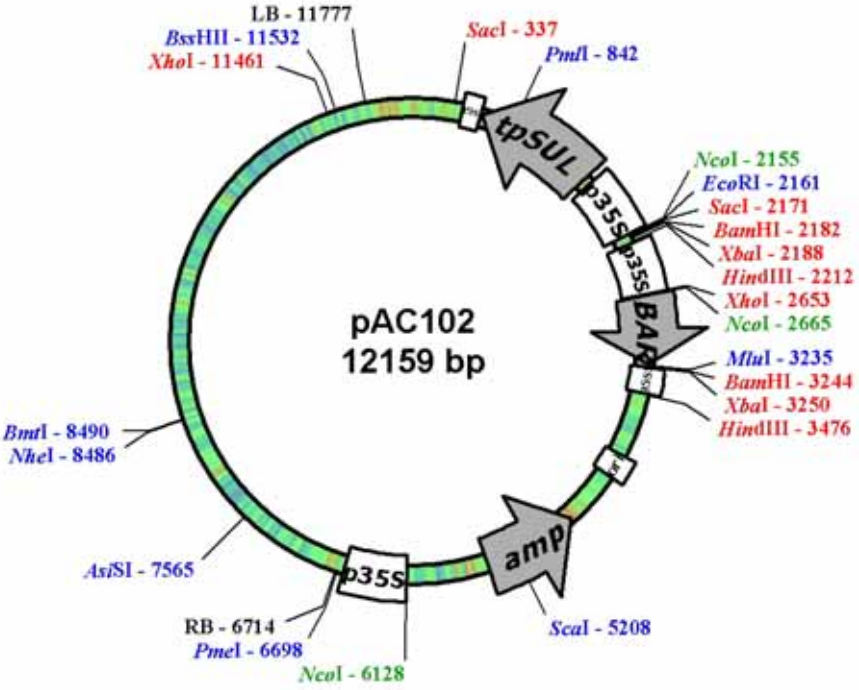

IV
II
VI
III
At
Os
Gm
Hs
Mm
Rn
Ce
Dm
Sc
Sp
Ca


I
V
ZnF1
ZnF2
At
Os
Gm
Hs
Mm
Rn
Ce
Dm
Sc
Sp
Ca

H Plasmids

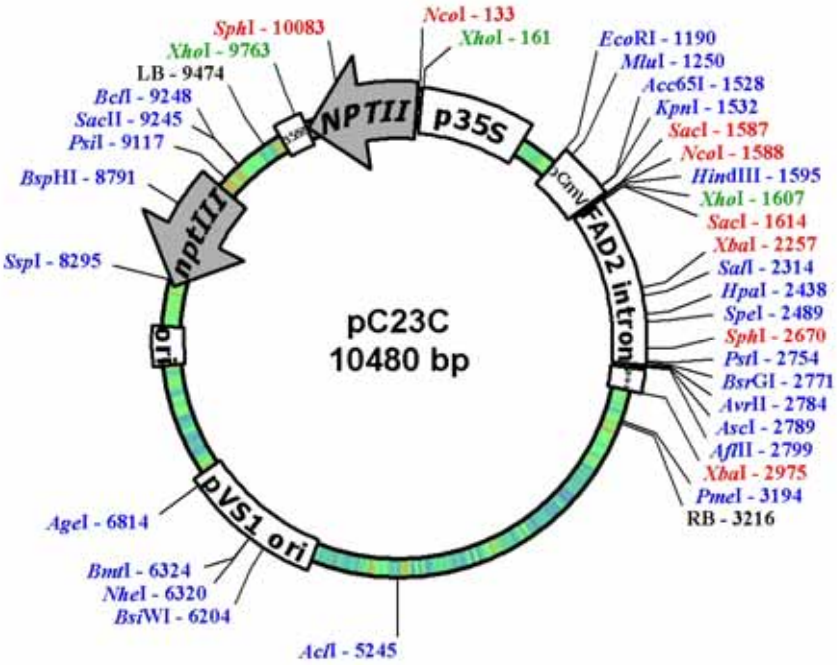
Plasmid 1: Map of pAC102

Activation-tagging vector



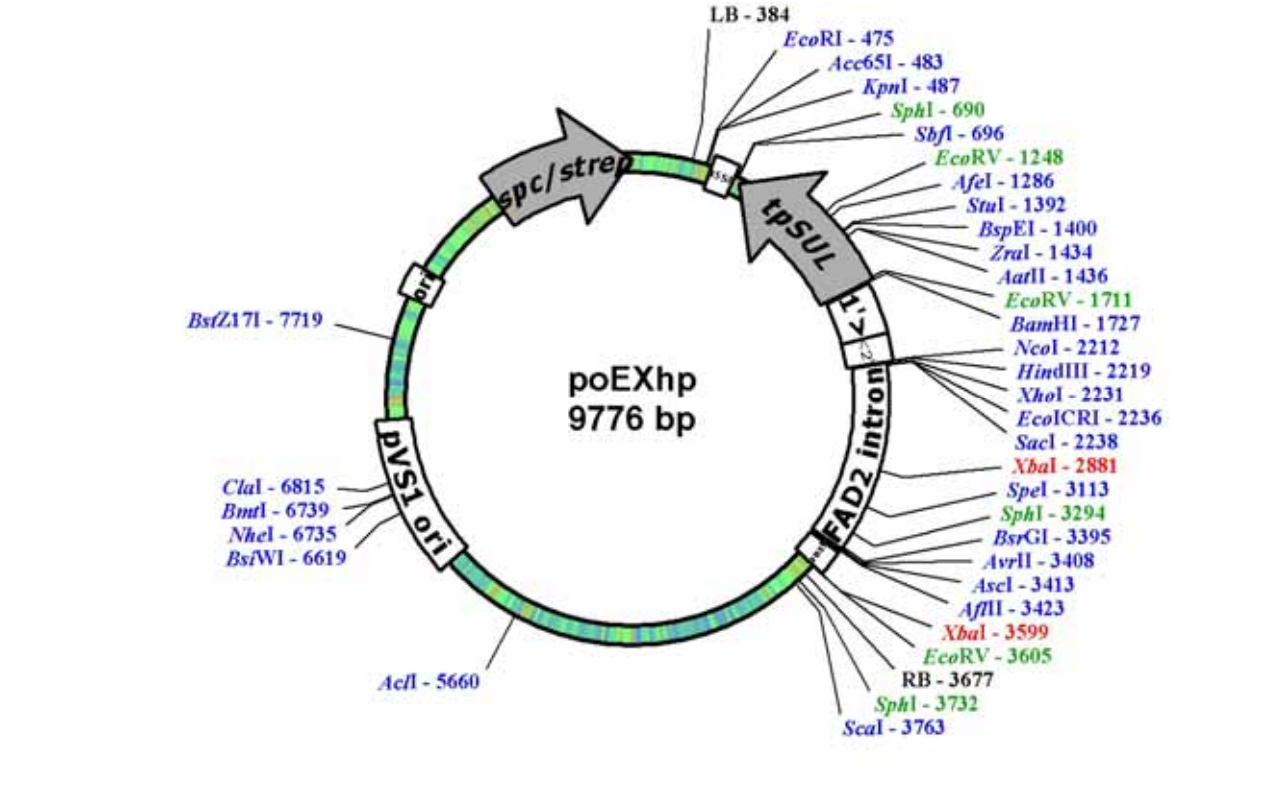
Plasmid 2: Map of pC23C

Vector for complementation experiment: *POLδ1* cDNA was cloned into the *Hin*dIII and *Avr*II sites



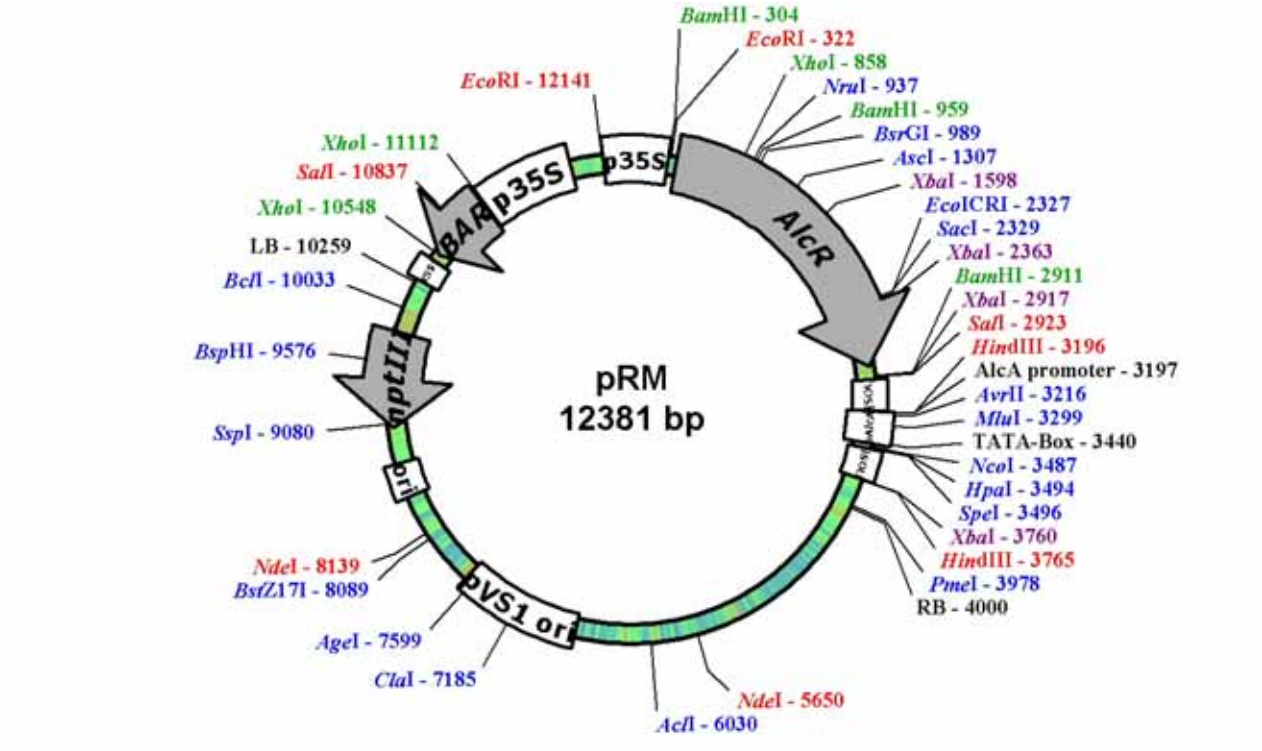
Plasmid 3: Map of poEXhp

Vector for c-RNAi lines: *POLδ1* cDNA fragment was cloned into the *Nco*I and *Xho*I sites and into the *Bsr*GI and *Avr*II sites in sense and in antisense orientation, respectively.



Plasmid 4: Map of pRM

Vector for i-RNAi lines: the *POLδ1* hairpin construct of poEXhp (see above) was cloned into the *Nco*I and *Spe*I sites.



CURRICULUM VITAE

Name	SCHÜRMANN David
Date of birth	15th August 1972
Nationality	Swiss

Education

2000-2005	PhD studies at the Friedrich Miescher Institute under the supervision of Prof. Dr. Barbara Hohn, Prof. Dr. Ueli Grossniklaus (University of Zürich) and Prof. Dr. Holger Puchta (University of Karlsruhe) "Genetics of DNA replication and homologous recombination in Arabidopsis"
1998-1999	Scientific trainee at the Institute for Plant Sciences, ETH Zürich under the supervision of Dr. Bruno Tinland and Prof. Dr. Ingo Potrykus "Characterization of an Arabidopsis gene putatively involved in the switch from mitosis to meiosis"
1998	Diploma thesis at the Institute for Plant Sciences and the Institute for Biotechnology at the ETH Zürich under the supervision of Prof. Dr. Ingo Potrykus, PD. Dr. Christof Sauter and PD. Dr. Pauli Kallio "Expression of the *Vitreoscilla* hemoglobin gene in wheat"
1993-1998	Studies of Agronomical Sciences, ETH Zürich
1992	"C-Matura" (Swiss equivalent of A-levels), Kantonsschule Sursee LU

Publications:

Schuermann D, Fritsch O, Lucht J and Hohn B (in preparation). **Replication stress leads to genome instabilities in Arabidopsis DNA polymerase δ mutants.**

Schuermann D and Hohn B (in preparation). **A function of Arabidopsis *POLδ1* in DNA replication and repair by homologous recombination.**

Molinier J, Fritsch O, Schuermann D, Lucht J, Ries G and Hohn B (in preparation). **Genetic Screens for Altered Somatic Recombination in Arabidopsis**.

Schuermann D, Molinier J, Fritsch O and Hohn B (2005). **The Dual Nature of Homologous Recombination in Plants**. Trends in Genetics, 21(3), 172-181

Li A, Schuermann D, Gallego F, Kovalchuk I and Tinland B (2002). **Repair of Damaged DNA by Arabidopsis Cell Extract**. The Plant Cell, 14, 263–273